

It Was Just A B...

Science

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Breast Cancer

AAAS

SPECIAL SECTION

Breast Cancer

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Check out *Science Express*, the weekly podcast, videos, daily news, our research journals, and *Science Careers* at www.sciencemag.org.



COVER

Detail of *Wishing It Was Just A Bad Dream*, a 1995 painting by Hollis Sigler. Sigler's art was featured on the cover of the 7 October 1994 issue of *Science*, which reported the discovery of the breast cancer susceptibility gene *BRCA1*. She died of the disease in 2001. In this issue, *Science* examines breast cancer research 20 years after the cloning of *BRCA1*. See the special section beginning on page 1451 and at www.sciencemag.org/special/breastcancer.

Image: Courtesy of the Jean Howard and Joseph Virde collection, Salt Lake City, UT, and the Carl Hammer Gallery, Chicago, IL

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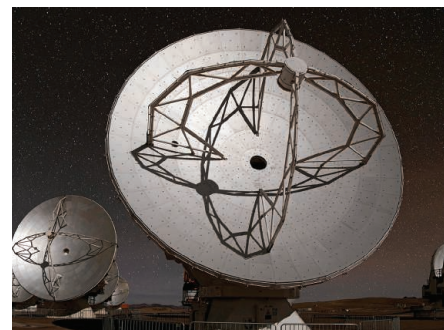
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G. J. Abel and N. Sander

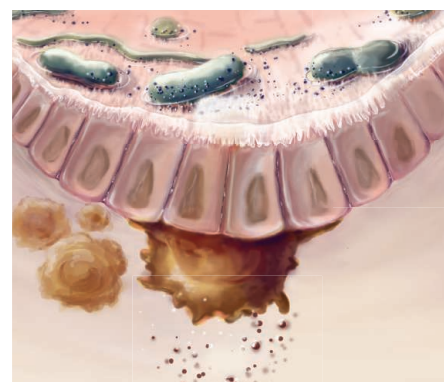
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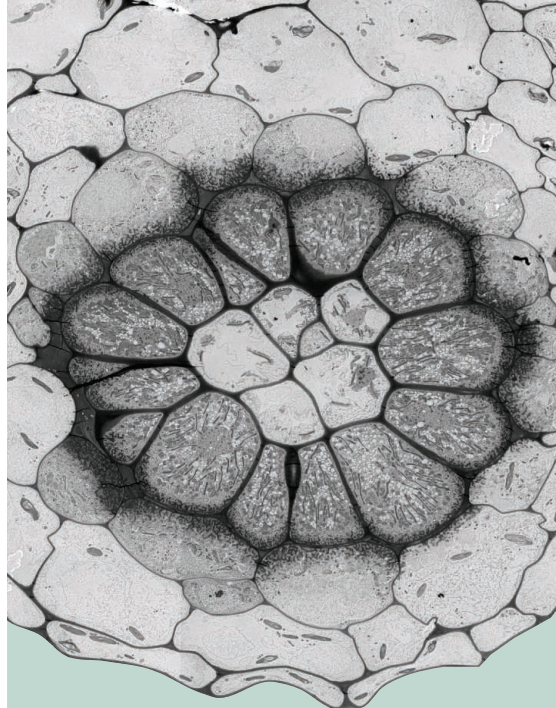
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From Drips to Tubes

In the evolutionary transition from aquatic to terrestrial habitats, plants acquired internal systems to transport water and provide structural support. **Xu et al.** (p. 1505, published online 20 March) studied a family of genes and the cells they control to better understand the innovations required to adapt to dry land. In *Arabidopsis*, specific transcription factors regulate development of xylem—the plant tissue that transports water. The moss *Physcomitrella patens* has similar genes, which regulate development of hydroids and stereids, cells specialized in water transport and structural support. The similarity in the genes and their functions suggests the evolutionary origins of land-plant vascular systems.

Beyond Brownian Motion

On long time scales, the random Brownian motion of particles diffusing in a liquid is well described by theories developed by Einstein and others, but the instantaneous or short time scale behavior has been much harder to observe or analyze. **Kheifets et al.** (p. 1493) combined ultrasensitive position detection with sufficient data collection to probe the Brownian motion of microbeads in fluids on time scales that are shorter than the characteristic bead-fluid interaction time.

Look After the Child

Investing in children has been demonstrated to improve their lives, both during the school-age years and afterward, as assessed by outcomes such as employment and income; furthermore, these investments often help those in the most need. **Campbell et al.** (p. 1478) report that these investments can also lead to improved adult health. Results from a randomized and intensive intervention that involved 122 children in four cohorts recruited in the 1970s suggest that full-day child care for the first 5 years of life has produced adults in their 30s with better metabolic and cardiovascular health measures.

Gut Immune Tolerance

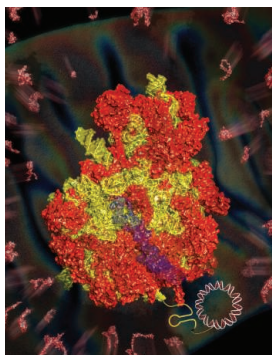
With the constant assault of food antigens and its billions of resident microbes, the gut is an important site of immune tolerance. By studying specific intestinal immune cell populations in genetically modified mice, **Mortha et al.** (p. 1477, published online 13 March; see the

Perspective by **Aychek and Jung**) found that gut macrophages produce the cytokine interleukin-1 (IL-1) in response to signals derived from the microbiota. IL-1 acts on type 3 innate lymphoid cells in the intestine, which then produce the cytokine, colony-stimulating factor 2 (Csf2). Csf-2, in turn, induces myeloid cells (including dendritic cells and macrophages) to produce regulatory factors like retinoic acid and interleukin-10, which support the conversion and expansion of regulatory T cells, a population of cells known to be critical for maintaining immune tolerance in the gut.

Mitoribosomes

Mitochondria—found in all eukaryotic cells—have transferred most of their genes to the nuclear genome.

The nuclear-localized mitochondrial genes are expressed and translated in the cytoplasm and the resulting mitochondrial proteins are imported into the mitochondria. Nevertheless, a few genes remain within mitochondria in the mitochondrial genome, and these genes are translated by mitochondrial ribosomes (mitoribosomes). **Amunts et al.** (p. 1485; see the Perspective



by **Kühlbrandt**) determined the structure of mitoribosomes from yeast using single-particle cryo-electron microscopy. The mitoribosome is highly diverged from the bacterial and eukaryotic ribosomes with, for example, a distinctive exit tunnel for the newly synthesized peptide, and a membrane facing protuberance that might help to anchor the mitoribosome to the mitochondrial membrane.

One-Sided Story from Disk

In young analogs of the solar system, the ongoing erosion of comets and nascent planets produces dusty debris that is eventually expelled by the host star. Gas should also be released in this process when volatile ices sublime, but it is detected less often. Using the Atacama Large Millimeter/Submillimeter Array, **Dent et al.** (p. 1490, published online 6 March; see the Perspective by **Brandeker**) mapped a highly asymmetric disk of dust and carbon monoxide orbiting the planet-hosting star, β Pictoris. The distribution of gas and dust is consistent with two proposed scenarios: In one, an outward-migrating planet has resonantly trapped dust-yielding bodies in two clumps opposite the star. In another, the entire debris mass is the result of a single recent collision of Mars-sized bodies.

Controlling Quantum Plasmonics

Electron tunneling across cavities could potentially induce a quantum mechanical plasmon mode that would be important in nano-electronics, catalysis, nonlinear optics, or single-molecule sensing, but has been expected to occur only at length scales beyond the reach of current state-of-the-art technology. Using a system of plasmonic dimers comprising silver nanocubes bridged by a molecular self-assembled monolayer, **Tan et al.** (p. 1496; see the Perspective by **Nordlander**) observed quantum plasmonic tunneling between the resonators and were able to tune the frequency of this tunneling plasmon resonance via selection of the molecular tunnel junctions. Moreover, the effects were observed at length scales that are technologically accessible.

Hydrologic Thermostat

When the silicate-rich rocks and minerals in Earth's interior are uplifted and exposed to Earth's surface, they dissolve. On a geologic

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This Week in *Science*

Continued from page 1397

time scale, this chemical weathering process ultimately creates a sink for CO₂, thereby influencing global temperatures. **Maher and Chamberlain** (p. 1502, published online 13 March) developed a theoretical framework for understanding the fundamental relationship between weathering, tectonics, and the geological carbon cycle. The analysis suggests that temperature plays less of a role in regulating chemical weathering—which is dependent on the balance of tectonic uplift and erosion—than runoff on continents and the time that silicate minerals are exposed to fluids. Plateaus in weathering fluxes with increasing runoff or temperature allows for the stabilization of atmospheric CO₂ despite high rates of uplift or erosion.

Optical Angular Selection

A monochromatic electromagnetic plane wave is typically characterized by three properties: its frequency, its polarization, and its propagation direction. While the selection of light signals based on the first two properties has been studied in depth, selection based on direction is relatively unexplored but equally important. **Shen *et al.*** (p. 1499) demonstrate a simple approach that provides narrow-angle selectivity over a broad range of wavelengths using heterostructured photonic crystals that act as a mirror for all but a narrow range of viewing angles where the crystals are transparent. Such angular selection should find a number of applications in, for example, high efficiency solar energy conversion, privacy protection systems, or high signal-to-noise detectors.



Move and Countermove

Receptors on plant cell surfaces are tuned to recognize molecular patterns associated with pathogenic bacteria. **Macho *et al.*** (p. 1509; published online 13 March) found that activation of one of these receptors in *Arabidopsis* results in phosphorylation of a specific tyrosine residue, which in turn triggers the plant's immune response to the phytopathogen *Pseudomonas syringae*. *P. syringae* counters by secreting a specifically targeted phosphatase, thus stalling the plant's immune response.

Keeping Germ Cells Special

Germ cells separate from the somatic lineage during early metazoan development. **Eun *et al.*** (p. 1513) found that *Drosophila* germ cells lose their identity and turn on somatic cell-specific marker expression in adult testes when a Polycomb group component E(z) is inactivated in somatic cells. However, only early-stage germ cells, including germline stem cells, retain this plasticity. Thus, one role for somatic cells in male gonads is to antagonize somatic identity in germ cells.

Circadian Rhythms

Circadian rhythms in the fruit fly *Drosophila* are driven by neurons in the brain. **Yao and Shafer** (p. 1516) analyzed different sets of neurons that can drive circadian rhythms. Manipulating the period of each set of neurons separately revealed that when the various clock signals were fairly consistent, the fly showed a robust circadian rhythm. But when the various clock signals were seriously out of sync with one another, the fly was oblivious to the day-night cycle.

Monitoring Migration

Migrant "stock" data—the number of people living in a country other than the one in which they were born—are frequently used to understand contemporary trends in international migration, but the data are severely limited. **Abel and Sander** (p. 1520) present a set of global bilateral migration flows estimated from sequential stock data in 5-year intervals. The percentage of the world population moving over 5-year periods has not shown dramatic changes between 1995 and 2010. People from individual African countries tended to move within the continent, whereas people from Europe tended to move to very diverse locations.

CREDIT: WEISHUN YU AND YUHAO ZHANG



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Breast Cancer—Thinking Globally

FEW DISEASES HAVE SEEN MORE RAPID SCIENTIFIC PROGRESS OVER THE PAST THREE DECADES than breast cancer. Beginning in the 1980s, screening mammography led to sweeping improvements in early cancer detection. At the same time, endocrine treatment and systemic chemotherapy proved life-saving. The development of drugs that target hormone receptor and *HER2/neu* oncogene signaling pathways, coupled with biomarker-based subclassification of the disease, have helped make breast cancer therapy a more precise science. Cloning of the *BRCA* genes provided insight into inherited predisposition and the opportunity for genetic testing. As a result of these advances, breast cancer death rates in the United States dropped by 34% between 1990 and 2014.

That is good news, but not for everyone. Breast cancer is a global disease (see the News feature on p. 1452). It is the world's most common cancer among women, the most likely reason that a woman will die from cancer, and is becoming an increasingly urgent problem in low- and middle-income countries (LMICs). Of the 19.7 million cases projected to occur in the next decade, 10.6 million will be in LMICs. By 2020, over 1 million cases per year will occur in LMICs alone, where the majority of breast cancer deaths already occur. In LMICs, a large fraction of women with breast cancer are diagnosed with advanced-stage disease and have no access to treatment or basic palliative care. How can we transform existing knowledge about early detection, diagnosis, and treatment into clinical practice in these countries?

Advanced-stage cancers require aggressive, expensive, and resource-intensive treatment protocols that are not minor to the systems that must provide them or to the patients who undergo them. Optimal therapies from wealthy countries cannot be fully implemented in LMICs because of substantial resource constraints. Therefore, a priority in LMICs should be to improve early detection, which has its own set of economic, cultural, and political barriers to implementation. In 2002, the Breast Health Global Initiative (BHGI) was established, bringing together a diverse global group of clinicians, public health scientists, international health experts, health policy-makers, social scientists, and economists to develop evidence-based strategies for breast cancer early detection, diagnosis, and treatment using systematic analyses of resource utilization and projected outcome improvements. The resulting resource-stratified guidelines (RSGs) provide a comprehensive tool set to evaluate capacity for breast health delivery, identify critical missing resources, and define prioritization schemes for increasing capacity. For early detection, networks must be devised connecting tertiary cancer centers with regional hospitals and surrounding clinics where initial patient contact takes place. For treatment, special infrastructure may be needed. Tamoxifen is an affordable, highly effective generic drug for treating estrogen receptor (ER)-positive breast cancer, but the ER testing that is required to determine which cancers have the potential to respond is often unavailable in LMICs. Used effectively, RSGs can provide a platform for policy-makers to prepare for breast cancer's rising tide.

High-income countries debate the value of early detection through mammography in part because it has led to overtreatment of a subset of patients. This discussion has limited relevance for most LMICs, which lack the infrastructure to make population-based screening mammography a realistic option. The 25-year update of the Canadian National Breast Screening Study was widely publicized for finding no survival benefit for women who underwent screening mammography.* Largely overlooked, however, was the success of the early detection strategies used in the study's control group, where favorable outcomes were achieved through breast health education combined with annual clinical breast examination. The study's control group methodology could serve as a model for program development in LMICs for early detection.

Adaptations are necessary to span the gap of inadequate health care capacity. The tragedy of global breast cancer is not that we don't know what to do—it is that we don't know how to get it done.

— Benjamin O. Anderson

10.1126/science.1253344

*A. B. Miller *et al.*, *BMJ* 348, g366 (2014).



ASTRONOMY

Study in Contrasts

In order to image exoplanets directly, it helps to increase the contrast between the extremes of the bright stellar host and any faint companion. Close *et al.* report the use of an advanced adaptive optics system to observe HD142527, a young star that hosts a disk of gas and dust, whose well-studied morphology includes a large gap that sparked discussion of active planet formation there. The authors confirm the presence of a companion at only 12 times the Earth-Sun distance, detecting it in visible light emitted by gas accreting onto it. This lower-mass stellar companion orbits the primary star just at the inner edge of the gap, implying partial responsibility for clearing its neighborhood, just as planets are thought to do in other cleared disks. At the specific wavelength of light where gas accretion releases energy, the brightness difference between the two bodies is less pronounced than at other wavelengths, where the difference instead depends largely on their extremely distinct temperatures. The detection of this accreting companion in visible light should encourage more searches for planets that are still accumulating gas in their formation phase, when observers can take advantage of the favorable contrast levels. — MMM

Astrophys. J. Lett. **781**, 10.1088/2041-8205/781/2/L30 (2014).

observations suggest that GPR161 dysfunction contributes to the development of triple-negative breast cancers. — PAK

Proc. Natl. Acad. Sci. U.S.A. **10.1073/pnas.1320239111** (2014).

PHYSICS

Frustrated Magnetism

In magnetic materials, ions positioned on the lattice sites attempt to minimize their energy by aligning their spins; in some cases, however, the configuration of the lattice makes it impossible to minimize the local energy on all sites at the same time—a situation known as geometrical frustration. Besides spin, charge and orbital degrees of freedom can also be associated with frustration. In the compound CuIr_2S_4 , which has Ir ions of mixed valence, a charge order sets in below 230 K with alternating octamers of Ir^{3+} and Ir^{4+} , and a spin-singlet ground state has been predicted to occur. Kojima *et al.* use a technique sensitive to magnetism, muon spin rotation spectroscopy, to detect a glass-like disordered paramagnetic state below ~ 100 K instead of the expected spin singlet. The authors find that the magnetism is associated with Ir^{4+} ions in specific locations and that even a minute substitution of Cu by Zn (which also notably reduces the charge order) leads to its disappearance. As this small substitution, equivalent to a single hole per 12 Ir octamers, influences the electronic state in such a dramatic way, the authors hypothesize the existence of interoctamer correlations, which may stabilize the glass-like ground state. — JS

Phys. Rev. Lett. **112**, 087203 (2014).

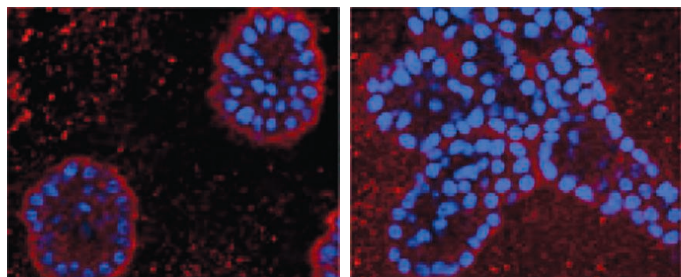
CANCER

Negative Reinforcement

About 15 to 20% of breast cancers are classified as “triple negative,” so called because these tumors do not express three key proteins that are biomarkers and/or drug targets for breast cancer: the estrogen receptor, the progesterone receptor, and HER2 (a member of the epidermal growth factor receptor family). Triple-negative tumors are aggressive and more likely to

metastasize than other breast cancers, and there is no effective treatment. To acquire new insights into the biology and possible therapy of these tumors, Feigin *et al.* looked for aberrant expression of G protein-coupled receptors, cell signaling proteins that have been successfully targeted for treatment of other disorders such as depression. An orphan receptor called GPR161 was found to be overexpressed in triple-negative but not in other breast cancer types. In cell culture, high expression levels of GPR161

induced proliferation of mammary epithelial cells, disrupted the acinar structures formed by these cells, and enhanced their invasive capacity. GPR161 was shown to activate the mTORC1/S6K signaling pathway. These



EDUCATION

Owning the Experience

An essential component of a successful undergraduate research experience is project ownership. Currently, measurements of project ownership take place on a project-based level at individual institutions, with no real protocol for standardization or the integration of data into a larger database. Hanauer and Dolan address this issue with the development of the Project Ownership survey (POS). Three main components—specification of the undergraduate research experience, assessment of degrees of project ownership, and emotive scales—form the basis of the Web-based POS. Fully analyzed to determine its dimensionality, reliability, and validity, the 16-item POS showed high internal consistency and was able to differentiate between students

Continued on page 1407

Continued from page 1405

who studied in traditional versus research-based laboratory courses, making it a valuable tool that can be used to further assess the features of an undergraduate research experience that may encourage students to pursue a degree in science. Furthermore, wide use of the POS may enable the scientific community to characterize the broad range of research experiences taking place across institutions, allowing for the identification and proliferation of successful features of undergraduate research experiences that enhance project ownership. — MM

CBE Life Sci. Educ. **13**, 149 (2014).

NEUROSCIENCE

Neurons' New Birthday

Mice generate new olfactory neurons throughout adult life. Although humans seem not to generate new olfactory neurons in adulthood, the site at which those new neurons are born, the lateral ventricle wall, seems to generate neurons in both mice and humans. Ernst *et al.* use carbon-14 dating from nuclear bomb test exposures to compare the birth dates of neurons to the birth dates of the people being analyzed. The results show that unexpectedly, some striatal neurons are generated after birth. Calculations indicate that neurogenesis from the lateral ventricle wall in the adult human supplies interneurons in the striatum. — PJH

Cell **156**, 1072 (2014).

GENETICS

Tweaking a Switch

Transcription factors regulate gene expression by binding to specific chromosomal operator sites.

Many transcription factors are repressors, with transcription turned off when the repressor is bound.

A simple operator occupancy model assumes that the level of repression is determined only by the equilibrium binding of the repressor to its operator. Hammar *et al.* have now used a single-molecule chase assay to directly test this in living cells. They measured the time that the lac repressor protein LacI remained bound to the natural lacO₁ operator and to a stronger, artificial lacO_{sym} operator. It is assumed that transcription is turned off during this time, so this is termed τ_{off} . They also measured the average time that the operators remained unbound so that transcription can be on (τ_{on}). The repression ratio in the simple occupancy model would be given by $RR = (\tau_{\text{on}} + \tau_{\text{off}})/\tau_{\text{on}}$. The calculated repression ratios were

compared with repression ratios measured based on an enzymatic reporter assay, thus monitoring protein expression rather than repressor binding. There was agreement for the lacO₁ operator, but for the lacO_{sym}, more repression was seen than would be expected based on a simple occupancy model. This could be accounted for either by promoter-specific cooperative interactions between LacI and RNA polymerase or simply by transcription initiation driving the system out of equilibrium; fast transcription initiation could lead to the synthesis of transcripts before the repressor has equilibrated with DNA. Such effects need to be considered in examining mechanisms of gene regulation. — VV

Nat. Genet. 10.1038/ng.2905 (2014).

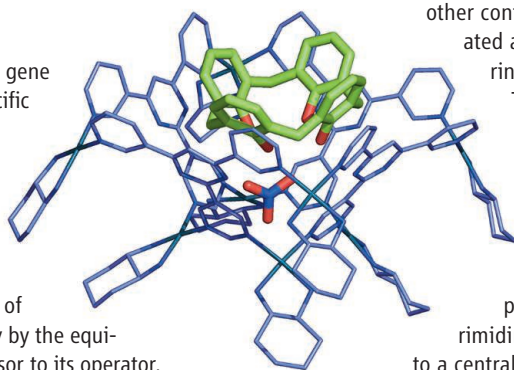
CHEMISTRY

Accommodating Hosts

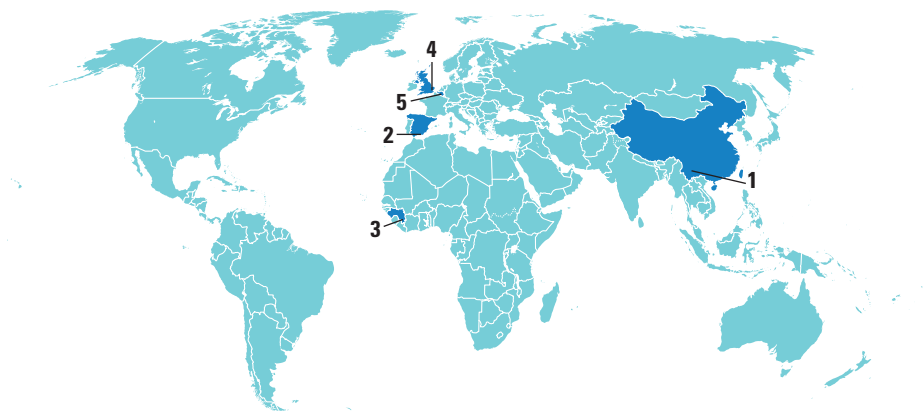
The binding of smaller, more rigid guest molecules by larger, more flexible host biomolecules (such as substrates and enzymes) can lead to induced fit: changes in the host structure to accommodate the guest. However, the binding of two large biomolecules can involve structural changes in both partners and is called mutually induced fit. Sawada *et al.* report a synthetic system in which two small molecules both act as hosts and make structural changes during binding. One host, calix[4]arene, has four phenyl rings that can form a conformer that has a cone-shaped binding surface, but this structure

is in rapid equilibrium with other conformers created as the phenyl rings flip down. The other host was formed from a triangular ligand L (formed from three pyridine and pyrimidine rings bound to a central pyrimidine ring) reacting with a Pd complex in aqueous solution. Two structures formed, mainly Pd₆L₃ trigonal pyramids (94%) but also Pd₈L₄ tetragonal pyramids (6%). When these two hosts were mixed in solution, the calix[4]arene shifted entirely to the cone structure, and the second host shifted entirely to the minority tetragonal pyramid structure to create an extensive binding surface. Furthermore, this host trapped a nitrate ion, which neither host alone could do. — PDS

J. Am. Chem. Soc. 10.1021/ja500376x (2014).



AROUND THE WORLD



Yunnan province, China 1

New Virus Suspected In Chinese Deaths

Research into the deaths of three men in southwestern China has turned up an intriguing new viral species. The men contracted severe pneumonia and died in June 2012 after removing slag from a derelict copper mine. Analysis of anal swabs from rats in the mine revealed a potential killer: a



On the scene. Infectious disease researchers outside the cave mine in Yunnan.

virus resembling those in the *Henipavirus* genus, scientists reported in the June issue of *Emerging Infectious Diseases*.

Two of the three confirmed henipavirus strains are deadly to humans, and all three appear to circulate in the wild in fruit-eating bats called flying foxes. Bats and shrews in the mine in Yunnan tested negative for the new virus, named Mojivang paramyxovirus (MojV); only rats were infected. MojV “could be a ‘bridging’ virus between those in bats and rodents,” says Lin-Fa Wang of the Australian Animal Health Laboratory in Geelong. A survey of

38 bat species across China prompted by the MojV find failed to turn up any henipaviruses. http://scim.ag/_henipa

Almería, Spain 2

Struggling Observatory Loses Leader

A tight budget plan imposed by the funders of the German-Spanish Astronomical Center at Calar Alto (CAHA) has driven the center’s director to resign. José María Quintana plans to step down in late April because the Spanish National Research Council and Germany’s Max Planck Society, which jointly operate the observatory, support a funding strategy he considers too harsh.

An operating plan signed in May 2013 set aside €1.6 million per year until 2018 for CAHA, whose Potsdam Multi-Aperture Spectrophotometer has been used to survey nearby galaxies. When Quintana assumed the post in June 2013, he advocated budget increases that he says would have kept the center’s three telescopes running and retained core staff. But he announced his resignation in late January when the funders opted to keep post-2014 funding flat. Spanish media reports say that since then, CAHA’s interim managers have fired cooking and cleaning staff and restricted the operation of one telescope by 10 days a month. While still hopeful for the observatory’s future, Quintana foresees an era of “unreasonable minimums.” http://scim.ag/_CAHA

Southeastern Guinea 3

Ebola Outbreak Kills Dozens

The first large human outbreak of Ebola hemorrhagic fever in West Africa has killed dozens of people in Guinea and may have moved into neighboring Liberia and Sierra



Responders. Health workers unload medical supplies in Guinea earlier this week to deal with Ebola.

Leone. On 25 March, the World Health Organization said that 86 cases with 59 deaths had been documented in four districts in southeastern Guinea, and at least six suspect cases, with five fatalities, were being investigated across the border in Liberia. The culprit is the Zaire ebolavirus (ZEBOV), says virologist Sylvain Baize of the Institut Pasteur in Lyon, France, who with his colleagues analyzed initial samples from the outbreak. ZEBOV is the deadliest of the five known Ebola species.

This is the first outbreak of ZEBOV outside Central Africa, and Baize says it will be important to try to understand how the outbreak started. Fruit bats have been found to be asymptomatic carriers of the virus, and they are strongly suspected as a reservoir where Ebola hides between outbreaks, but, Baize notes, the field studies to find a definitive culprit “are quite difficult.”

London 4

U.K. Budget Prioritizes Big Data, Cell Therapies, and Graphene

In a 2014 budget statement, U.K. Chancellor of the Exchequer George Osborne announced £222 million (\$366 million) in new funding for research projects. Forty-two million pounds will go to create the Alan Turing Institute for Data Science, which will focus on the analysis and application of big data. Another £74 million will help set up a center to manufacture cell therapies for clinical trials and a lab to research new technologies using graphene. And £106 million will go toward 20 new centers for doctoral training, Osborne announced on 19 March.

While researchers have largely welcomed the new funding, the Campaign for Science and Engineering (CaSE) points out that the core science budget, which has

remained stagnant since 2010, devotes only 1.72% of Britain's gross domestic product to research and development—significantly less than many other developed nations including Germany, the United States, and Japan (all around 3%), and the European Union average of 2.08%. The flat budget “is biting scientists and engineers and squeezing universities,” and may deter outside investment, CaSE Director Sarah Main said in a statement.

Brussels 5

World Map of Environmental Conflicts Launched

Want to see which communities around the world are fighting off a project, such as a mine or a dam, that could harm the environment? A new online “atlas of environmental justice,” developed by a group of academics and activists funded by the European Commission, allows just that. Activists, scholars, or the purely curious can search and filter the data across 100 fields, including by commodity, company, country, and type of conflict.

The atlas, launched last week, was built by Environmental Justice Organisations, Liabilities and Trade (EJOLT), an E.U.-funded project that brings together 23 teams from academic and activist organizations. It now lists 915 conflicts; 271 involve local scientists and professionals, and 17% are described as environmental victories. “It’s only the tip of the iceberg,” says EJOLT spokesman Nick Meynen. The organization aims to raise the coverage to 2000 conflicts in the coming year, which would enable quantitative analyses. Academics can provide input to expand the range of cases and fill the gaps—including blank spots in China. <http://ejatlas.org/>

NOTED

>The World Health Organization (WHO) was set to **declare India and the 11-country Southeast Asia region polio-free** on 27 March. Long the global epicenter of the virus, India saw its last case on 13 January 2011. That leaves two more WHO regions to go: the Eastern Mediterranean Region, where Afghanistan and Pakistan are the roadblocks, and the African region, where Nigeria remains endemic and has sparked nearby outbreaks.



Champions of the Sargasso

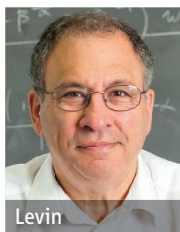
It may lie beyond the jurisdiction of any nation, but the Sargasso Sea—and the hundreds of marine species it supports—has many advocates. Last week, a team of 33 researchers with the Danish Eel Expedition 2014 arrived in this open-ocean region of the North Atlantic to study the declining European eel populations that spawn there. Using fine-mesh nets (pictured above) to catch larvae, they hope to “plug the main gaps in our knowledge” of the eel’s early diet and breeding habits, says the project’s leader, Peter Munk of the National Institute of Aquatic Resources at the Technical University of Denmark in Charlottenlund. The team also will examine whether climate-related changes to ocean currents are contributing to the eel’s decline.

Meanwhile, five countries, including the United States and the United Kingdom, signed a nonbinding agreement earlier this month to protect the area from overfishing and pollution. The organization behind the agreement, a partnership between scientists, conservation groups, and the government of Bermuda called the Sargasso Sea Alliance, released a film celebrating the agreement, available at <https://vimeo.com/89868953>.

NEWSMAKERS

Tyler Prize Goes to Ecologist With a Mathematical Eye

Whether he’s looking at a colony of mussels or the international balance of political power, Princeton University ecologist **Simon Levin** sees the same thing: complex systems. This week, Levin was named the 2014 recipient of the \$200,000 Tyler Prize for Environmental Achievement for a 4-decade career that connects mathematical



concepts such as game theory to the dynamics of animals and ecosystems.

Levin’s work has influenced Pacific salmon recovery efforts and protection of the Hudson River, but it has also found some unlikely applications in topics beyond environmental science, from the destructive dynamics of cancer cells to global affairs in the age of terrorism.

Levin says he’s encouraged that a complex, interconnected view of ecosystems is triumphing over more “simplistic” management schemes. “What you’re really trying to preserve are the broad, emergent features of the system,” he says, “whether it’s a forest system or a financial system.” >>

>>NEWSMAKERS

Biomedical Donors Leave Research Legacy

Two of science's most generous philanthropists recently passed away. On 19 March, **Patrick McGovern**, who became one of American's wealthiest people after founding the publisher International Data Group, died. McGovern received an undergraduate biophysics degree at the Massachusetts Institute of Technology in 1959 and 4 decades later, he and his wife donated \$350 million to create a neuroscience research institute at his alma mater.



McGovern



Stowers

James Stowers, who died on 17 March, originally studied medicine before moving into business and making his fortune running an investment firm. In 1994, after both had run-ins with cancer, he and his wife established the independent Stowers Institute for Medical Research in Kansas City, backing its endowment with more \$2 billion in assets. The institute opened in 2000 and now has nearly 400 scientific staff members.

BY THE NUMBERS

7 million Deaths worldwide in 2012 as a result of indoor and outdoor air pollution exposure, which amounts to one in eight total deaths, according to a new World Health Organization estimate.

Random Sample**DNA's Forgotten Discoverer**

James Watson and Francis Crick may be the names most associated with DNA, but a German biotech company is determined that the world never forgets Friedrich Miescher, the Swiss scientist who first discovered the molecule, nearly a century before the pair famously deduced its structure.

CureVac, a company based in Tübingen, Germany, that develops RNA-based vaccines and therapies, won a €2 million prize for innovation from the European Union this month and announced plans to use some of that money to restore the University of Tübingen lab where Miescher made his discovery. Together with the university, the firm wants to transform the lab, the former kitchen of the old town's medieval castle, into a public exhibition about his work and legacy. (The university now uses the space as a computer room.)

While studying the composition of pus cells in 1869, Miescher found a molecule in cell nuclei that was not a protein, but a substance "not comparable to any hitherto known group." He intuited that the molecule, which he dubbed "nuclein," played a role in heredity, but didn't believe that a single molecule could lead to a huge variety of individuals and species, says Ralf Dahm, of the Institute of Molecular Biology in Mainz, Germany, who has written about Miescher's work. He says Miescher has kept a low historical profile, in part due to his "introverted" and "insecure" personality. "He's one of the most important guys in science," says CureVac CEO Ingmar Hoerr, "and not many people are aware of it."

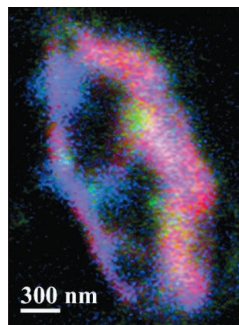
FINDINGS**Spacecraft Nabs Specks Of Pristine Stardust**

For the first time, scientists have laid their hands on primordial material unaltered by the violent birth of the solar system. The precious sample—containing seven interstellar dust particles returned to Earth by the Stardust spacecraft—weighs just a few trillionths of a gram.

In 2000 and 2002, Stardust barreled through deep space with its tennis racket-like dust collector panel extended to catch particles of dust in centimeter-thick blocks of aerogel. The trick was to slow and

retain the particles, traveling at 15,000 kilometers per hour or more, without vaporizing them. Back on Earth, 30,714 volunteers—the "dusters" of the Stardust@home project—examined microscopic images taken within the aerogel to pick out telltale tracks left by speeding particles. One hundred million searches later,

seven "probable" dust impacts on the collector have emerged, NASA scientists reported last week at the Lunar and Planetary Science Conference in The Woodlands, Texas. Next, they must transfer the infinitesimal specks of dust from inside the aerogel into instruments for further analysis. http://scim.ag/_stardust





Seeing red. Mine runoff has turned the Hengshi River in southern China dark red and has tainted cropland in Shangba, downstream.

ENVIRONMENTAL SCIENCE

China Gets Serious About Its Pollutant-Laden Soil

SHANGBA, CHINA—He Youtian feels old beyond his years. The 57-year-old farmer is hobbled by kidney stones, as is his wife, who is recovering from uterine cancer. In his youth, He recalls, this lush corner of southern China's Guangdong province was "beautiful and fertile." Then, about 4 decades ago, an open-pit copper and sulfur mine began operating upstream on the Hengshi River, turning the water dark red and tainting irrigated fields. Nongovernmental organizations have designated Shangba as a "cancer village," one of perhaps hundreds of areas in China so pollution-drenched that cancer rates are rumored to be off the chart.

China's economic ascent has littered the land with environmental disaster zones like Shangba. The government now appears eager to tackle the problem—but without fully acknowledging its scale to the public. In December, the environment ministry announced that 3.33 million hectares of cropland—2.5% of China's arable land—is too contaminated to grow food safely, according to a national soil survey conducted from 2006 to 2010. Yet the details of the survey were so alarming, sources say, that the central government declared them a "state secret."

In a speech at the National People's Congress this month, Premier Li Keqiang declared a "war on pollution" and ticked off aggressive measures to curb air pollution

and clean up tainted lakes. Soil pollution is also now in the spotlight. The latest 5-year plan singles out five industries—mining, smelting, e-waste, tanneries, and chemical plants—as egregious soil polluters and sets a target to reduce, by 2015, discharges of lead and other heavy metals by 15% from 2007 levels. Last week, the environment ministry reportedly began hammering out a plan to curb all sources of contamination by 2020 and to begin remediating filthy areas. "The new government doesn't want soil pollution to become a serious social problem and cause unrest," says Pan Genxing, a biochemist at Nanjing Agricultural University.

But the secrecy surrounding the survey data has some researchers fuming. "People have the right to know if they're exposed to soil pollution risks," says Chen Ruishan, a geologist at Hohai University in Nanjing. He also worries about the scientific underpinnings of any cleanup effort. Withholding data, he says, hampers efforts to devise remediation strategies.

Other scientists suspect the survey itself was inadequate, based on the few details that have leaked out. The survey plotted contamination on 8-square-kilometer grids—"too large to be meaningful," says Chen Nengchang, a soil scientist at the Guangdong Institute of Eco-Environmental and Soil Sciences in Guangzhou. Soil pollution, he says, is "highly localized" and

depends, for instance, on particular irrigation sources farmers tap. By comparison, he notes, soil surveys in Japan use 160-square-meter plots.

Shangba's ills hint at the magnitude of the problem China faces. When Chen first visited the reputed cancer village in 2005, he was shocked by the Hengshi River's red hue, lent by heavy metals leaching into the water from the state-owned mine upstream. But the most serious threat to human health, Chen says, is a colorless contaminant: cadmium.

Cadmium "moves easily through the food chain, from soil to humans," Chen says. Rice, a staple crop, is especially prone to absorb the heavy metal, which accumulates in the liver and kidneys and is a proven public-health threat, having been linked to itai-itai disease, a mass poisoning in Japan starting in the early 20th century. Last May, Guangzhou's government revealed that 44% of rice it tested in provincial restaurants—rice grown in Guangdong and in neighboring Hunan province—contained elevated cadmium levels. The news ignited public outrage and hurt farmers' incomes.

When Chen tested crops in Guangdong for cadmium in 2008, he found a telling discrepancy that could point to a cleanup strategy. Rice grown in the dry winter had as much as 10 times the maximum amount of cadmium that China deems safe for human consumption, but rice grown in waterlogged soil during the summer rainy season contained only slightly elevated levels. The explanation is soil acidity. In winter, the soil pH tends to be lower, which releases more cadmium ions for rice and other plants to

absorb. That suggests that irrigating winter fields at critical times of crop development could raise soil pH and lower cadmium uptake, Chen says.

China needs to implement such cleanup measures on a large scale, experts argue. With a fifth of the global population and only 7% of the world's arable land, "China cannot afford to simply retire farmland," says Xin Song, an environmental engineer at the Chinese Academy of Sciences' Institute

of Soil Science in Nanjing. Interim measures that could help sustain farms plagued by heavy metals, Pan says, include "decreasing metal uptake by plants." One approach is to spread biochar—biowaste baked in a kiln—on cadmium-contaminated fields. Pan and colleagues found that adding biochar to tainted fields in Jiangsu province raised soil pH and reduced cadmium uptake up to 50% in rice and 30% in wheat. But such efforts could be hampered by decades of overuse of

inorganic fertilizers, which have ratcheted up soil acidity across China.

In Shangba, authorities tried to help residents by building a rainwater reservoir for drinking water and for irrigation, but it is "not enough," He says. Farmers here must continue to rely on water from the Hengshi River—and risk tainting the food they grow—until China rises to the herculean task of cleansing its befouled land.

—CHRISTINA LARSON

ENVIRONMENT

25 Years After the *Exxon Valdez*, Where Are the Herring?

Twenty-five years ago this week, the oil tanker *Exxon Valdez* slammed into Bligh Reef in Alaska's Prince William Sound, causing what was, at the time, the largest oil spill in U.S. waters. At least 250,000 barrels of crude created a slick that stretched 750 kilometers and killed an estimated 250,000 seabirds, 2800 sea otters, 22 killer whales (orcas), and billions of salmon and herring eggs. Exxon made numerous payments to settle legal claims that resulted from the 24 March 1989 wreck, including a \$900 million civil penalty dedicated to ecological restoration and research. A quarter-century later, that settlement con-

Q: How is the wildlife of Prince William Sound doing?

W. S. P.: A number of species are considered recovered. Fairly recently, [biologists declared] sea otters and harlequin ducks recovered. Those are the last two big ones. Then there is one pod of orcas that is on its way to extinction. [The pod, one of eight resident in the sound, had 36 members at the time of the spill.] Essentially, the pod no longer has viable females and is never going to recover.

There are some species that are "unknown," and they are going to continue to be unknown because there just wasn't enough baseline information to ever be able to truly evaluate if they have recovered. Some birds, including Kittlitz's murrelets and marbled murrelets, are in that category. And some fish.

Q: Herring is still a big unknown, right?

W. S. P.: Yes, herring is a really interesting case. The population collapse occurred 3 years after the spill. In the winter of 1992–93, [fishers]

were expecting a large fishery, but when they went out less than 25% of the fish were there, and they appeared lethargic and diseased. There are definitely some different opinions as to why it collapsed. It could have been a combination of nutritional stress and oil exposure. We know levels of zooplankton [which herring eat] were low after the spill, and that the herring had direct pathways for oil exposure, which could have weakened their immune system.

One thing that makes it complicated is natural variability. Herring have a boom-bust cycle, and we know that in the past the [Prince William Sound] stock has collapsed and recovered. Before the spill we were running 80,000 to 100,000 tons of spawning stock. Now, we've been bouncing around just below 22,000 tons. That level is what the local community cares about; once we exceed it, the fishery can reopen.

Q: What are some other active research questions?

W. S. P.: A major one is lingering oil. If you go out and know what you are looking for, you can still find oil trapped within the beach. Dig a hole and it comes out of the sediment in droplets; it appears to be fresh. It looks like the [degradation] rate is between zero and 4% annually, and 4% is at the extreme high end. Studies suggest it isn't surfacing at quantities that have a large-scale ecological impact, but it is definitely out there.

We really need to figure out the mechanics of oil entrapment, and how it moves through sediments. It's a really important question because if you don't understand that, we don't know whether we can get the oil out of the sediment, or get it out without causing more harm than good.

Q: What's next?

W. S. P.: We're putting a lot of work into understanding natural variability so we can better understand the impacts of something like a spill. Officially, [the settlement fund] kicked off a 20-year-long monitoring program 2 years ago, and we already have some records that date back 25 years. So, hopefully, one legacy of this is that researchers will have some very long-term records of the ecosystem that will help us understand how it is changing.



Innocent bystander. The 1989 *Exxon Valdez* oil spill killed an estimated 250,000 seabirds and oiled many more, such as this one.

tinues to pump some \$3 million a year into spill-related science, with plans to continue extensive monitoring through 2032. Oceanographer W. Scott Pegau helps oversee those programs as the research program manager of the Prince William Sound Oil Spill Recovery Institute in Cordova, Alaska. He spoke with *Science* about what we do and don't know about the ecosystem's recovery. (This interview has been edited for brevity and clarity.)

—DAVID MALAKOFF

CREDIT: JACK SMITH/AP IMAGES

HUMAN EVOLUTION

Oldest *Homo sapiens* Genome Pinpoints Neandertal Input

SITGES, SPAIN—In 2008, Siberian ivory carver Nikolay Peristov was searching for ancient mammoth tusks eroding from the banks of the Irtysh River in western Siberia, when he found fossilized bones instead. Back in his workshop in Omsk, he showed the bones to local paleontologist Aleksey Bondarev, who recognized a human thighbone. Bondarev in turn showed it to an anthropologist friend, and it was passed on up the chain to some of the world's top experts in human evolution. They dated it to 45,000 years ago, making it one of the oldest known modern humans in northern Asia and Europe.

Now, the bone has opened a window on the genetics of our species at a crucial moment: soon after their arrival in northern Eurasia. At a meeting* here last week, paleogeneticist Svante Pääbo of the Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany, announced that his team has sequenced the thighbone's entire nuclear genome to high accuracy—an astonishing 42x coverage. “This is by far the oldest sequenced genome of a modern human,” he said.

The as-yet unpublished bone and genome are yielding new insight into when moderns interbred with our extinct cousins, the Neandertals. “Genetically we now have a modern human that just barely postdates the Neandertal introgression into modern humans,” explains Bence Viola of the Max Planck, who was not at the meeting but has studied the bone, found near the village of Ust-Ishim.

Geneticists have sequenced the complete genomes of even older Neandertals as well as those of a third kind of Stone Age human from Denisova Cave in Siberia. But the DNA of ancient members of our own species has been elusive, partly because it is hard to rule out contamination from people who handled the bones. Now, high-resolution sequencing techniques can distinguish ancient genomes from current ones, researchers say. The next oldest genome of a *Homo sapiens*, announced last fall, comes from a boy who died 24,000 years ago near the Siberian village of Mal'ta (*Science*, 25 October 2013, p. 409).

Nearly twice as old as the Mal'ta bones,

the Ust-Ishim femur shows that almost as soon as modern humans arrived in northern Eurasia, they made themselves at home in harsh climates, Viola says. “Ust-Ishim is at about the latitude of Stockholm or Juneau, Alaska,” he notes. “It was not that much warmer at the time, so they must have been quite well adapted to northern environments.”

were the ones that mingled. But the team's analysis favors a more recent rendezvous. The femur belonged to an *H. sapiens* man who had slightly more Neandertal DNA, distributed in different parts of his genome, than do living Europeans and Asians. His Neandertal DNA is also concentrated into longer chunks than in living people, Pääbo reported. That



Stone Age genomics. Researchers have complete genomes from three types of humans who coexisted during the last Ice Age: Neandertals (orange); Denisovans (blue); and *Homo sapiens* (yellow).

In his talk, however, Pääbo zeroed in on one issue: interbreeding between Neandertals and modern humans. Scrutiny of the genomes of living people and ancient Neandertals has shown that 1% to 3% of the DNA of Europeans and Asians comes from Neandertals. But how extensive the original mixing was, and where and when it happened, have been the subject of debate.

Modern humans moved from Africa into the Middle East as early as 120,000 years ago, according to fossils of archaic modern humans at Skhul and Qafzeh caves in Israel. But they apparently skirted the colder climes of Europe and northern Asia until much later, about 40,000 to 45,000 years ago. Neandertals, meanwhile, arose somewhere in Europe or Asia; they occupied the same caves as modern humans in the Middle East and Denisova, although at different times, and both groups overlapped in Europe from about 30,000 to 45,000 years ago.

Because all living people in Europe and Asia carry roughly the same amount of Neandertal DNA, Pääbo's team thought that the interbreeding probably took place in the Middle East, as moderns first made their way out of Africa. Middle Eastern Neandertal sites are close to Skhul and Qafzeh, so some researchers suspected that those populations

indicates that the sequences were recently introduced: With each passing generation, any new segment of DNA gets broken up into shorter chunks as chromosomes from each parent cross over and exchange DNA. Both features of the Neandertal DNA in the femur suggest that the Ust-Ishim man lived soon after the interbreeding, which Pääbo estimated at 50,000 to 60,000 years ago.

Researchers still think the Middle East is a likely place for the encounters. Other fossils in Israel, such as a 49,000-year-old Neandertal at Tabun Cave, might belong to people who were alive at the time of the unions or just after, says archaeologist Ofer Bar-Yosef of Harvard University, who was not a member of the team.

Pääbo's team is now analyzing the Siberian man's genome to see just what genes he inherited from Neandertals. They have already found one: the Neandertal form of a gene that shapes the curvature of the spine. Evolutionary geneticist Sarah Tishkoff of the University of Pennsylvania says she's eager to learn what else the genome reveals. “It's like having a time machine, to go back 45,000 years to see how the genomes of modern humans differed both from our own and also from Neandertals and Denisovans.”

—ANN GIBBONS

*Cell Symposium: Evolution of Modern Humans - From Bones to Genomes, 16–18 March.



Womb for improvement. Mats Brännström (center) announced the first mother-to-daughter womb transplants at a 2012 press conference.

MEDICINE

Pioneering Womb Transplant Trial Highlights Risks and Ethical Dilemmas

GOTHENBURG, SWEDEN—When Turkish surgeon Ömer Özkan announced that he had carried out a successful womb transplant in 2011, it was a difficult day for Mats Brännström, the gynecologist and obstetrician who had led the Uterine Transplantation Project at the University of Gothenburg's Sahlgrenska Academy here since 1999. "They took us by surprise," concedes Brännström, who believed he lacked real rivals in the race to bring the first healthy baby from a transplanted womb into the world.

Özkan's patient has since been pregnant twice, but both pregnancies ended in a miscarriage, so Brännström believes he could still be first. But he and his colleagues hope their efforts will also be remembered for something else: scientific rigor. The Swedish team has carried out most of the animal research underpinning womb transplantation, and last month, Brännström published a detailed paper about his first nine human womb transplants, carried out between late 2012 and early 2013. Seven transplant patients still have their new wombs and are menstruating normally, the team reported in *Fertility and Sterility*; four have already had the first embryos placed inside them.

"It is safe to say that the Swedish effort is monumental," says Giuseppe Del

Priore, the national director of gynecologic oncology for Cancer Treatment Centers of America, who is based in Newnan, Georgia. But transplanting a womb poses unique challenges, in part because of the rigors of pregnancy, and the procedure is fraught with ethical dilemmas. Medical ethicist Ruby Catsanos of Macquarie University in Sydney, Australia, who's working on a

"This isn't a lifesaving transplant; it isn't the same as a heart, lung, liver, or kidney transplant."

—NEIL HUBAND,
UK WOMB TRANSPLANTATION
RESEARCH TEAM

thesis about the subject, questions whether the risks of a massive surgery, especially for living donors, are justified for what is arguably a "quality of life" transplant.

Scientists hope that womb transplants can give women whose uterus is dysfunctional or missing—usually because of a congenital condition called

MRKH or cancer surgery—the option of bearing their own children. Many of these women have intact ovaries, so doctors can use standard in vitro fertilization (IVF) procedures to produce embryos from their eggs and a male partner's sperm, which can then be placed inside the transplanted womb.

The first documented human womb transplant was performed in 2000 by a surgeon in Saudi Arabia, but the uterus began to die after 3 months and had to be removed. Others besides Özkan and Brännström are interested as well; the UK Womb Transplantation

Research Team, for instance, aims to carry out four to five transplants as early as next year, in collaboration with Del Priore.

Brännström's group deserves credit because it "advanced carefully," says fertility researcher Kenny Rodriguez-Wallberg of the Karolinska Institute in Stockholm, first transplanting wombs in mice, rats, sheep, pigs, and baboons. In 2010, one of Brännström's rats became the first animal with a transplanted womb to produce a healthy offspring. That same year, Edwin Ramirez, a gynecologist and surgeon at St. John's Regional Medical Center in Oxnard, California, reported that a sheep with a donor womb gave birth to a normal lamb via cesarean section.

But experience in primates is scant. Brännström says his team did not attempt to impregnate its baboons because IVF techniques for that species aren't adequately developed. Japanese scientists removed the womb from a macaque monkey and then grafted it back; that animal delivered an extremely premature but apparently normal fetus after emergency cesarean section in 2011. (Human babies, too, would need to be delivered via C-section; the connection between the transplanted womb and the vagina can't dilate enough to let a baby through.)

One key question is how strongly to suppress the recipient's immune system after the operation and once she gets pregnant. (Because the womb would be removed after one or two successful pregnancies, the recipients won't have to take immunosuppressive drugs for life.) Some pregnant women already take immunosuppressive drugs, for instance because they have had an organ transplant; the resulting babies are at higher risk of premature birth and lower birth weight, but generally seem to do well. Brännström's team opted for a mild immunosuppression regimen to reduce side effects and the risk of postoperation infections. The Swedish team saw only minor signs of rejection, such as a buildup of white blood cells, in four of the seven recipients.

Brännström used living donors, in most cases the recipient's own mother. This allowed the team to plan operations well in advance and fly in top transplant surgeon Andreas Tzakis from the Cleveland Clinic to help with the surgery. But the U.K. team has already ruled out using living donors because it finds the risks unacceptable. "This isn't a lifesaving transplant; it isn't the same as a

heart, lung, liver, or kidney transplant,” says Neil Huband, a spokesman for the program. In many countries, women without a uterus could opt to have a surrogate mother bear their child—although not in Sweden, where surrogacy is banned.

The Swedish trial showed that the risks to the donor are real. To ensure a good blood flow and reduce the risk of thrombosis, the team opted to transplant not just the womb but also its long veins and arteries, which were attached directly to the large blood vessels deep in the recipient’s pelvis. This greatly complicated the donor operations, however;

they took 10 to 13 hours. In one “extremely difficult” case, the paper says, a 58-year-old donor developed a fistula between her urethra and her vagina, a condition which can lead to incontinence. She had to have a second round of surgery after 4.5 months.

Problems arose in two recipients as well: One had to have her transplant removed because of a uterine infection, the other after thrombosis. Catsanos, the ethicist, calls the complications “disconcerting.” “It just amplifies the problems that already exist,” she says. But Brännström believes the risks for the donor are manageable. “I would say

that for a novel procedure this complicated, there were few complications,” he says. And the operation time will come down to 5 or 6 hours once surgeons become more experienced, he says.

The Swedish team is now carrying out standard IVF treatment on the seven women. Brännström declined to discuss whether there have been any pregnancies so far, saying the publicity would put unnecessary pressure on the patients.

—RICHARD ORANGE

Richard Orange is a freelance journalist in Gothenburg, Sweden.

PLANETARY SCIENCE

Search for Martian Life Clears Another Hurdle

THE WOODLANDS, TEXAS—Members of the Curiosity Mars rover’s science team have announced a milestone in their search for signs of ancient life on Mars. After carefully checking recent results, the researchers said last week at the annual Lunar and Planetary Science Conference here, they are now reasonably confident that contaminants brought from Earth cannot entirely explain certain carbon compounds Curiosity spotted in martian rock.

If so, these simple organic compounds—chlorine-carrying methane and the like—either came with the tons of never-alive cosmic debris that sifts onto every planetary body or are something far more exciting: remains of martian life from eons ago, when a habitable lake and a rushing stream graced the rover’s now-dry landing site.

To reach that conclusion, scientists had to clear some daunting hurdles. First, they had to account for the effects of perchlorate, a corrosive, oxidizing compound that is pervasive on Mars and, when heated, can chew up any complex organic matter into little bits (*Science*, 12 April 2013, p. 138). They also had to reckon with known sources of contamination in Curiosity’s Sample Analysis at Mars (SAM) instrument package (*Science*, 31 August 2012, p. 1032). A chemical reagent brought along for future analyses of organics had somehow leaked into the system, and gases driven off soil and rock samples were reacting with a polymer used to prepare compounds for identification by mass spectrometry. As a result, the rover was making its own organics, including the small chlorinated methanes and the sizable compound chlorobenzene.

To sort out which of the device’s detections are truly martian, the team instructed SAM

to run blanks: analyses made with empty sample vials. They flushed both blanks and real samples with inert gas and warmed them to drive off the contaminating reagent. On Earth, they ran samples containing known organics through a SAM apparatus under Mars-like conditions. And on the rover, they tried experiments such as analyzing samples three times as big as normal to see whether the yield of particular organics would triple as well.

is martian as well. Or as one of his slides pointedly put it: “The detection of reduced organic compounds in Martian near-surface samples is a significant step.”

Some experts outside the 400-strong Curiosity team are more guarded. “The case for a martian source of carbon builds,” responded meteoriticist Mark Sephton of Imperial College London by e-mail. He was not at the meeting but read the four pages of abstracts describing the results.



Pay dirt. The Curiosity rover first drilled into martian rock at this spot on its way to 5-kilometer-high Mount Sharp. More drilling and much checking have yielded traces of martian organic matter.

At the meeting, Caroline Freissinet and Daniel Glavin of NASA’s Goddard Space Flight Center in Greenbelt, Maryland, and a score of fellow SAM team members reported that the latest results are persuasive. The new findings offer “compelling” evidence that some of the carbon-containing compounds SAM recently detected such as chlorinated methane, ethane, and propane came from organic matter in ancient martian rock, not from earthly contamination, Freissinet said. And Glavin said that other results are “a good indication” that some of the chlorobenzene

The case could build further if Curiosity strikes a richer vein of organic matter in future samples. Or SAM team members could try a more sophisticated technique to separate out the organic compounds in rock before the pervasive oxidizing agent chews them up. The next opportunity to drill a new sample will come within a few months when Curiosity is likely to pause on its way to its ultimate target, Mount Sharp.

—RICHARD A. KERR

With additional reporting by Emily Lakdawalla, a freelance journalist in Los Angeles, California.

SYNTHETIC BIOLOGY

Synthetic Biologists Design 'Living Materials' That Build Themselves

Organisms are master builders. Crabs assemble shells, corals amass reefs, and our own tissues build bone. Now, synthetic biologists are taking control of the construction process. Researchers in Massachusetts reported this week in *Nature Materials* that they've reprogrammed the genetic circuitry of bacteria to construct electronic and optical materials, complete with living cells in their midst.

The new materials can't yet compete with conventional electronic devices. Nevertheless, outside researchers say the feat opens a new door to using genetically engineered organisms to assemble complex materials from the ground up, with minimal help. "It's fantastic work," says Lingchong You, a biomedical engineer at Duke University in Durham, North Carolina, who was not involved in the research. Traditional manufacturing is typically energy-intensive, polluting, and often hazardous for workers. "But if we can harness the power of cells [to build structures], we can make the entire process 'green,'" You says. Moreover, because organisms are adept at engineering materials at many different size scales—the way our bodies imprint bone with structure on the nanoscale, microscale, and meter scale—the new work holds the potential for adding new levels of complexity to engineered materials.

The new work isn't the first foray into marrying engineered organisms with materials. In 1999, for example, Angela Belcher, a chemist now at the Massachusetts Institute of Technology (MIT) in Cambridge, and her colleagues engineered viruses to assemble semiconductor nanoparticles on their surfaces. Belcher's group has since gone on to program viruses to construct everything from electrodes for lithium-ion batteries and photovoltaics to catalysts that split water to generate hydrogen fuel. But because viruses don't harbor their own cellular machinery, the materials they make

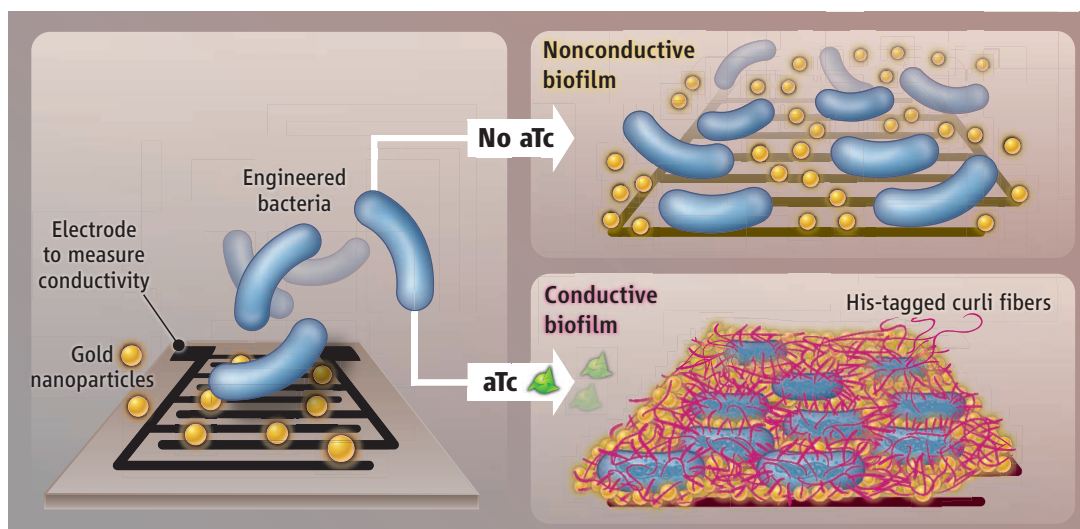
aren't alive. That means, for example, that they can't respond to their environment the way bacteria can: by organizing themselves in groups or healing themselves.

The new work, from Timothy Lu, a synthetic biologist at MIT, and colleagues, brings these previously disparate fields together. "Our idea is to put the living and nonliving worlds together to make hybrid materials that have living cells in them and are functional," Lu says. They started with *Escherichia coli* bacteria that naturally cooperate to produce sheetlike biofilms atop

film and latched on to gold nanoparticles that the researchers had sprinkled into their beakers, creating a network that conducts electricity (see figure, below).

By growing both batches of *E. coli* together, Lu's team could vary the composition of the film just by adding AHL and aTc at different times. In this case, the varying composition didn't add new function. But it sets the stage for doing so by binding other materials, for example. In a separate experiment, they used different peptide tags and chemical triggers to create bacteria that could trap tiny semiconductor particles called quantum dots, which altered the optical properties of the biofilm.

Lu now hopes to take advantage of recent advances in synthetic biology, in which researchers have programmed bacteria to



Filmmaker. When exposed to a chemical called aTc, bacteria produce fibers (pink) that cause them to attach to a surface and to one another. Amino acids called histidines on the fibers then grab gold nanoparticles, forming an electrically conducting film.

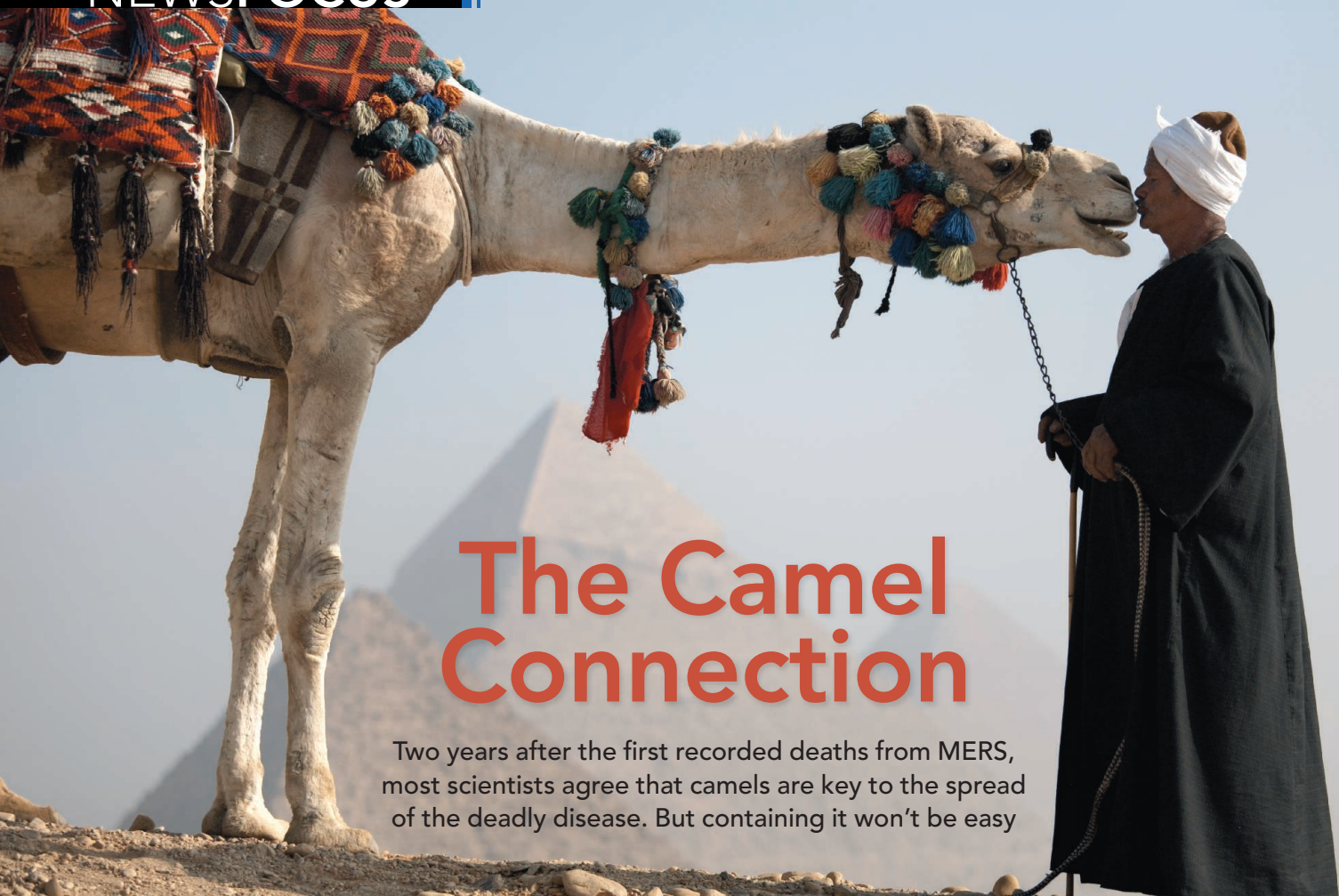
different surfaces. The bacteria bind these films together by secreting proteins called curli fibers. Made up of repeated protein subunits called CsgA, the fibers glue the bacteria to surfaces and to one another.

For their experiments, Lu's team first disabled the genetic pathway that allows bacterial cells to produce CsgA. They replaced it with an engineered genetic circuit that produces CsgA only when the researchers add a chemical trigger, a molecule called AHL. The team then engineered a separate batch of *E. coli* to produce altered CsgA tagged with short protein strands, or peptides, containing multiple histidine amino acids, which bind metal particles. These bacteria expressed the histidine-tagged CsgAs only in response to another chemical trigger, called aTc. When aTc was present, the bacteria settled into a

form colonies in the form of rings, bars, and other shapes. That could lay the groundwork for more complex architectures that could serve as electrodes, environmental sensors, and artificial tissues. Ultimately, the living materials might be fashioned into devices that repair themselves when damaged.

The technique might also be used to sponge up environmental toxins such as cadmium and recycle the material into complex optical and organic devices. It may even prove useful in prospecting: Designer bacteria, for example, might harvest gold from their environment and concentrate it in mats that could be scooped up. Such tests remain a ways off, however. Regulators would need to be convinced that engineered bacteria don't pose risks when released into the environment.

—ROBERT F. SERVICE



The Camel Connection

Two years after the first recorded deaths from MERS, most scientists agree that camels are key to the spread of the deadly disease. But containing it won't be easy

ON 21 MARCH 2012, A 25-YEAR-OLD STUDENT in Jordan started coughing. A few days later, he developed fever and shortness of breath. He was admitted to a hospital, where he ended up in intensive care. By the time he died on 25 April, several nurses and doctors had developed similar symptoms. One of them died, too. Then the mystery disease disappeared again.

Two years later, it's clear that the outbreak was the prelude to a protracted struggle against what is now known as the Middle East respiratory syndrome (MERS) virus. And although scientists still have lots of unanswered questions, evidence is mounting that camels play a key role in spreading the new pathogen. The camel-borne threat may extend far beyond the Middle East. Last month, a team led by Malik Peiris, an infectious disease researcher at the University of Hong Kong, showed that almost all camels in four Egyptian abattoirs had MERS antibodies in their blood; most had been imported from Ethiopia and Sudan, suggesting the virus may reside in large parts of Africa.

As a result, scientists are shifting their focus from human cases to camels. "It is

becoming ever clearer that MERS is a classic zoonosis," says virologist Christian Drosten of the University of Bonn in Germany. "We need to concentrate more on the animals." One idea gaining traction is to vaccinate camels. "Protecting camels right now may be the single most important thing we can do to protect humans," says Michael Osterholm, director of the Center for Infectious Disease Research and Policy at the University of Minnesota, Twin Cities.

But vaccines against coronaviruses, the group to which MERS belongs, are difficult to make, and researchers face hurdles ranging from money problems to the lack of a suitable animal model. Neither veterinarians nor health officials in the Middle East have risen to the challenge. To make matters worse, there are very few labs capable of safely studying deadly viruses in animals as big and unruly as camels.

The evidence mounts

After it was identified in June 2012, MERS quickly made its way to the top of the global public health agenda. The virus is related to severe acute respiratory syndrome (SARS),

a deadly pathogen that emerged in China in late 2002, killed 800 people around the world, and nearly caused a pandemic. The World Health Organization (WHO) still worries that MERS could be the sequel to that frightening episode. But while the disease has sickened at least 199 people and killed 84, it has largely been confined to the Middle East (see map, p. 1423).

The first clue that camels might be involved came from a patient from Abu Dhabi who died in a German hospital in March 2013. He owned racing camels and reported having had close contact with a sick camel before falling ill. Then in August, a team including Marion Koopmans, now at Erasmus MC in Rotterdam, the Netherlands, reported that 50 retired racing camels from Oman all had antibodies against MERS in their blood, which is evidence of a past or current infection. None of the cows, goats, and sheep they tested had such antibodies.

Some scientists were skeptical. Other coronaviruses known to infect domestic animals could have caused the positive tests, they argued. "We don't think camels have anything to do with it," said Ziad Memish,

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Beyond borders. A recent study found MERS in camels in Egypt, including animals imported from Sudan and Ethiopia.

an infectious disease specialist and Saudi Arabia's deputy health minister, at the time. "It just seemed so weird," says Peter Daszak, a veterinary epidemiologist at the EcoHealth Alliance in New York City. Genetically, "the virus looks like a bat virus." But later, MERS antibodies were reported in camels in Jordan, Egypt, the United Arab Emirates, and Saudi Arabia. And scientists investigating an outbreak on a farm in Qatar found snippets of the genome of the MERS virus in nasal swabs from three camels, suggesting that these animals were actually infected at the time.

Last month, a team led by Columbia University virologist Ian Lipkin reported evidence of a MERS infection in three-quarters of more than 200 Saudi Arabian camels tested; they also recovered MERS viral sequences that proved an exact match to viruses found in humans. Because the researchers rarely found the virus in camel feces, Lipkin believes fine droplets from the animals' noses transmit it. Some of the positive samples dated back to 1992, suggesting that isolated, unrecognized human MERS cases may have happened for decades, he says. And just last week, scientists from Saudi Arabia and Europe reported about a MERS patient who fell ill after caring for sick camels; they managed to sequence about 15% of the viral genome from one of the camels, and that part was almost identical to the sequence of the human virus.

No one is suggesting that contact with camels is the only way humans can become infected. Infected people can also spread MERS, as clusters of infection—such as the one in Jordan—indicate. Memish now accepts that camels seem to transmit MERS, but he says that some so-called index cases—patients who did not contract the disease from another patient—say they had no contact with the animals. It's unclear how they got infected.

Some may have been exposed indirectly, says Anthony Mounts, WHO's point person on MERS. In one recent instance, a man who worked with camels remained healthy, while his wife developed MERS. "The husband could have brought the virus home on his clothing," Mounts says, though he adds, "this is not proven at all." Contaminated food could be another route. But to identify all possible routes, local knowledge is crucial, Mounts says. He recently learned that drinking camel urine and applying it to the skin is used as a natural remedy for a variety of ailments in the region. The practice is probably not the pri-

mary route of transmission, he says, but it's something that should be studied.

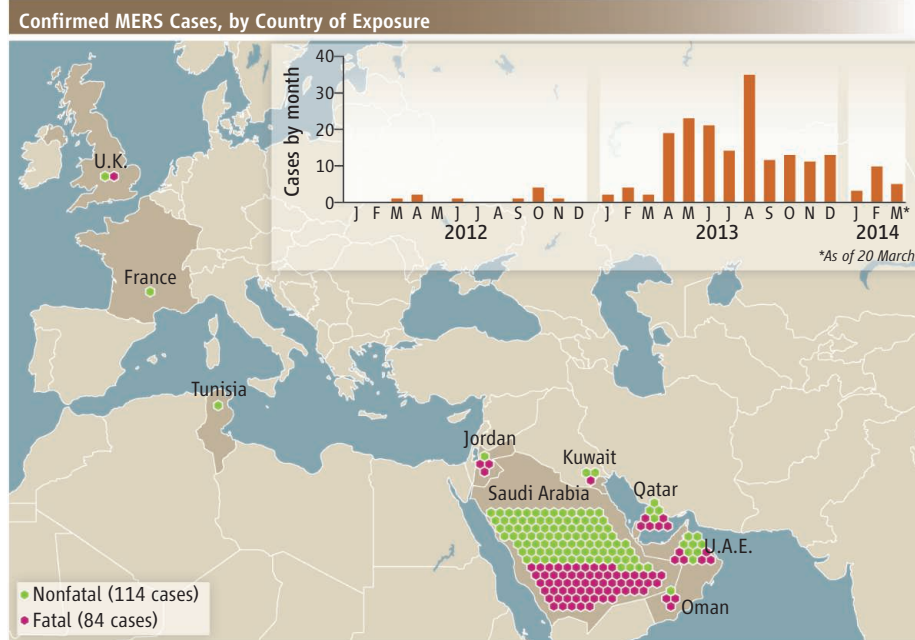
Other animals may still be involved. Bats seem to be MERS hosts, too; a fragment of viral RNA recovered from the feces of an Egyptian tomb bat living close to the first known Saudi MERS patient's house was identical to the patient's virus, so perhaps contact with bats can explain some cases. "It is important to remain open-minded about all potential sources of exposure for human and animal cases," a representative for the World Organisation for Animal Health writes in an e-mail.

If camels are MERS's main conduit, however, that puts the spotlight on countries far beyond the Middle East. Only about 260,000 of the world's 27 million camels live in Saudi Arabia. Africa has far more: 7 million camels in Somalia alone, 3 million in Kenya, and 1.5 million in Chad.

Beauty parades

For now, the MERS outbreak is generating a steady trickle of new cases: WHO reported four in January and four in February. But other zoonotic diseases have smoldered for years before they exploded, and no one knows whether MERS could also hold a nasty surprise. The fact that it didn't spread around the world after the Hajj, the massive pilgrimage to Mecca, in 2012 and 2013, has put some minds at ease. But Drosten says that may have been a lucky break. "Camels all give birth around the same time in the winter months," he says. They may contract MERS shortly after birth and be at their most infectious in spring. The Hajj is now in autumn, but because the Islamic year is shorter than the calendar year, it will eventually occur in the spring, Drosten says, "and that may change everything."

If camels are the problem, vaccinating them might be the solution. A camel vaccine



Regional problem? All known MERS cases so far occurred in the Middle East or were linked to that region.

The virus probably circulates in these herds as well, Peiris says. His 27 February paper in *Emerging Infectious Diseases* presented some early evidence. The scientists tested 52 camel blood samples from four abattoirs around Egypt and found antibodies against MERS in 48 of them, mostly from camels imported from Sudan and Ethiopia. They also took nose swabs from 110 camels and found MERS RNA in four imported animals. It's very likely that infection has gone unnoticed in Africa, both in animals and humans, Peiris says. "Health authorities really need to test patients with severe pneumonia all across Africa for MERS."

would be far cheaper to make than a human one, because testing in animals is faster and easier. Keeping the animals healthy would also make economic sense because camels are valuable, Daszak says: "They're eaten; they're raced; there are beauty parades. The Saudi government is going to have to act on this." Breeding facilities would be the place to deploy a vaccine, says microbiologist Matthew Frieman of the University of Maryland School of Medicine in Baltimore. "We have an opportunity to vaccinate all newborn camels to block infection from older camels, which seems like it's what's happening," he says.

But it's not that simple. Camel breeders don't see MERS as a problem because it does not seem to make camels very sick, and perhaps not at all, says Juan Lubroth, chief veterinary officer of the Food and Agriculture Organization of the United Nations in Rome. Nor have veterinarians and animal health officials shown great interest. "I have not been very successful in engaging my peers at the national or regional level," Lubroth says. Most countries don't see MERS as an immediate threat, and the U.S. National Institutes of Health has not yet awarded any grants for vaccine studies. Microbiologist Shibo Jiang of the New York Blood Center in New York City says he could not stir up any interest among companies either, "because of the unpredictable market in the future."

Nor does anyone know how to make a MERS vaccine. Jiang believes the best target may be the virus's spike, a protein sitting on the surface that it uses to attach to cells. He and his colleagues fused a 212-amino acid piece of the spike to the stem of a human antibody. This vaccine caused mice to produce antibodies that protected cells in a petri dish from infection.

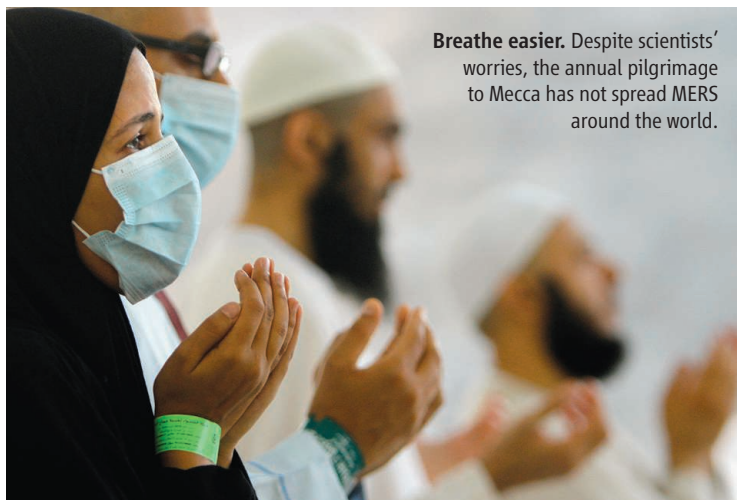
But coronavirus vaccines pose special challenges. One worrisome example is feline infectious peritonitis, a fatal cat disease. Cats carrying antibodies against this coronavirus are known to get sicker, instead of being protected. Fears that this might happen in humans, too, have plagued attempts to develop a SARS vaccine based on a whole, killed virus. The exact mechanism isn't quite clear, but Jiang found that with SARS, using just the part of the spike protein that binds to the cell receptor avoided the problem. He hopes the same will hold true for MERS.

But the receptor-binding area can change rapidly as the virus evolves, Frieman says, which might limit the protection offered by a vaccine. He has tried another approach: In collaboration with a company called Novavax, Frieman produced insect cells engineered to express the whole MERS spike protein. He harvests the protein as nanoparticles, small clumps of molecules sticking together. When these were injected into mice, the animals produced antibodies against MERS, Frieman reports in an as-yet unpublished paper.

A third strategy comes from Gerd Sutter of the Ludwig Maximilians University in Munich, Germany, who engineered a small-

pox vaccine strain called MVA to carry the genetic information for the spike. The vaccine might kill two birds with one stone: It would act as a MERS vaccine in the animals but should also protect them against camelpox, making it more interesting to camel owners.

Testing vaccine candidates is the next hurdle. A good animal model for MERS has been hard to develop; the spike protein binds to a protein called DPP4 on the surface of human lung cells, but the murine version of DPP4 is so different that MERS does not infect mice. Rhesus macaques seem to become infected but show hardly any symptoms: "Without a CT scan it's hard to tell that these animals are ill," says Lisa Hensley of the National Institute of Allergy and Infectious Diseases' lab in Frederick, Maryland. Marmosets get sicker, but they are hard to come by. Other groups have tried mice, ferrets, hamsters, and guinea pigs; none of them worked very well.



Breathe easier. Despite scientists' worries, the annual pilgrimage to Mecca has not spread MERS around the world.

So microbiologist Stanley Perlman of the University of Iowa has engineered an animal model: He packaged the human form of DPP4 into an adenovirus and then infected mice with it, coaxing them into expressing the protein on their cells' surfaces. The animals could then be infected with MERS, Perlman reported in a paper this month. How closely such a model mirrors what happens in camels or humans is unclear, however.

In fact, scientists still know disappointingly little about what MERS does in camels, says virologist Bart Haagmans of Erasmus MC. It's still unclear whether infected camels get sick, and at what stage they are most infectious to other animals or to humans. "There are a lot of questions that need to be answered in camel experiments," Peiris says. Haagmans says he would love to do those studies, but Erasmus MC doesn't have facilities to house the animals. Few labs around the world do.

Politics instead of science

Many other important questions about MERS remain unanswered, and scientists say that's in part because the affected countries have been uncooperative from the start. Are many people infected without showing symptoms? Are some people more susceptible to the disease than others? "The only people who can really answer those questions are people in Saudi Arabia, where that information resides," Lipkin says.

Mounts has long tried to set up a so-called case-control study in the affected countries to understand what patients have in common. "We need to agree on a series of questions about animal exposure, environmental exposure, and compare the answers with controls of a similar age and the same sex," he says. But the idea has been met with resistance in the region; an early March meeting with scientists and government

officials in Riyadh didn't help. "Public health is rarely about the science. It's usually the politics that stand in the way," Mounts says.

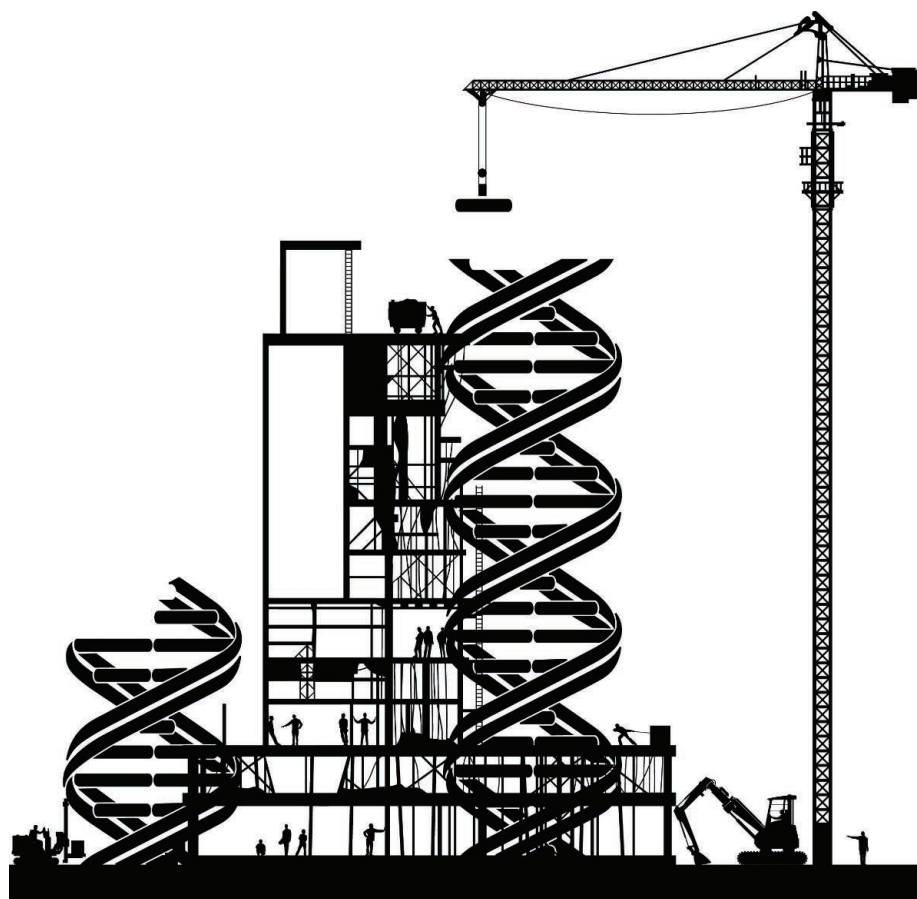
Memish, the Saudi deputy health minister, disputes that his country is dragging its feet. He says he's interested in working with any scientist, provided they don't have financial or business interests in MERS. One problem is that the disease has a stigma attached to it, Memish writes in an e-mail: "Nobody wants to take part in anything related to MERS," making it "difficult to get a 6-page, very

detailed questionnaire filled by a grieving family member." Some patients' families even moved away to avoid media attention and health officials' questions, Memish writes; questioning enough index cases could take "some years," he adds.

Still, not a single lung section of a MERS patient has ever been published, Frieman notes. "No one has any idea what this thing does in humans," he says. "It blows my mind."

The lack of progress may matter only to a handful of patients and scientists, if MERS continues to remain largely confined to its camel hosts. But SARS, too, lurked in the shadows for years, until it picked up the ability to transmit more easily from one human to the next—and threatened the world with a pandemic. If that happens with MERS, Osterholm says, "we'll all look at ourselves and say 'Why didn't we do more?'"

—KAI KUPFERSCHMIDT



Building the Ultimate Yeast Genome

Chromosome by chromosome, a global army of researchers and students is putting together the first synthetic eukaryote genome

When geneticist Ronald Davis first suggested a decade ago that his colleagues try to create artificial yeast chromosomes and install them in a living cell, Jef Boeke didn't think much of the idea. Davis, who is at the Stanford University School of Medicine in California, was known as a visionary. He proposed that a lab-made yeast would be the next step in the then-emerging field of synthetic biology. But Boeke couldn't see the point of replicating what nature had already made, especially because designing and synthesizing a 12.5-million-base genome seemed onerous, or even impossible. As Boeke listened to Davis's talk at a major yeast genetics meeting in 2004 in Seattle, he says, "I remember thinking 'Why on Earth would you want to do that?'"

How times have changed. Boeke, a geneticist who recently moved to New York University Langone Medical Center in New York City, and his colleagues have just finished the first complete synthetic yeast chromosome and are well on their way to

putting together several more, thanks to technological advances in manufacturing DNA and a global army of collaborators, mainly undergraduate students.

Other researchers have synthesized a bacterium's full genome, but the yeast job is orders of magnitude more complex. For starters, *Saccharomyces cerevisiae* has 16 chromosomes compared with the one in bacteria. Yet if the effort by Boeke and his army succeeds, it should offer broad benefits. "It gives us the ability to fully explore the yeast genome," Davis says. "If you really want to understand an organism, you should be able to design or redesign one."

Commonly called baker's, brewer's, or budding yeast, *S. cerevisiae* is vital for many food and drink industries, from beer to bread. Genetic engineers have already tweaked it in myriad ways for many uses, such as making ethanol. Recently it's been put to work building an antimalarial drug, artemisinin, and its potential for churning out other key chemicals is slowly being realized.

Yeast is also a workhorse for biologists probing basic cellular and metabolic processes in eukaryotes. Back in 1996, it became the first eukaryote to have its genome deciphered, and since then yeast geneticists have knocked out every gene and done analyses of all the interactions among them and their encoded proteins. For a long time, it was the only organism in which biologists could readily mutate specific DNA bases, as they found it easily incorporates foreign DNA through a process called homologous recombination.

The synthetic genome under construction by Boeke's army will be the ultimate modification. When it's done, Sc2.0, as some call it, will not be just any ordinary yeast strain. In designing Sc2.0, Boeke and his colleagues streamlined the typical yeast genome and built in sites that will make it possible to reshuffle the genome at will, potentially yielding more desirable properties and helping biologists figure out what each gene does. The endeavor "is bold, imaginative, and is going

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to teach us a lot about what the constraints are for synthesizing a whole genome and what constraints there might be relative to genes and chromosomes,” says Jasper Rine, a yeast geneticist at the University of California, Berkeley. “It will definitely serve as a landmark in the development of synthetic biology,” adds Chantal Shen, a collaborator at BGI in Shenzhen, China.

Until recently, however, no one knew if a truly synthetic chromosome could even sustain eukaryotic life. And when Boeke first attempted to construct a small chunk of yeast chromosome 9—a mere 90,000 bases—few had tried to work with a piece of DNA that big. During the project’s earliest days, the effort threatened to fizzle out.

But companies are now able to make ever bigger pieces of DNA, and labs from several different countries are now sharing the labor of synthesizing yeast chromosomes. Those developments, on top of his most recent success, make Boeke hopeful that live yeast containing the whole synthetic genome will be replicating in his lab within 4 years. “We are making history,” says Sc2.0 collaborator Junbiao Dai, a molecular biologist from Tsinghua University in Beijing.

Rough beginning

Boeke traces his change of heart about the synthetic yeast project to 2006, while he was still at Johns Hopkins University in Baltimore, Maryland. Over coffee, his Johns Hopkins colleague Srinivasan Chandrasegaran was trying to convince him to make a large number of zinc-finger nucleases. These DNA-modifying enzymes are Chandrasegaran’s specialty, and a toolkit full of them would make the yeast genome easier to manipulate. Boeke wasn’t that interested and, almost as a joke, suggested a more drastic way to take control of the yeast genome: synthesize the whole thing. To his surprise, Chandrasegaran jumped on the idea, and the pair brought Joel Bader, a computer scientist at Johns Hopkins, into the discussion.

Despite Boeke’s dismissal of Davis’s proposal just 2 years earlier, the trio quickly concluded that making an artificial yeast genome would, in fact, be worthwhile if it could be a testbed for learning about the genome itself. They decided to start with the 90,000-base “R” arm of chromosome 9—the shortest in the yeast’s genome—and spent a year arguing about how the synthetic sequence should differ from the natural one. The trio considered just including the genes they wanted, “but we quickly realized it would be very risky to eliminate whole bunches of genes” without really knowing

in advance what the effect of that loss on the whole system would be, Boeke recalls.

So they started with the natural sequence of the chromosome 9 arm and instead added DNA to it that would enable them to induce changes at will. They inserted short sequences of DNA called loxP just after the end of each nonessential gene—those they knew could be knocked out or changed without killing the yeast. They also put loxP sites near significant landmarks, such as the telomeres at the tips of chromosomes and the centromeres at each one’s center. LoxP is a part of a standard molecular biology tool. When activated by a chemical added to cells, it kicks off a chromosomal version of musical chairs. The result: genomic rearrangements and new yeast strains with different properties. Boeke and his colleagues called this system SCRaMBLE, for synthetic chromosome rearrangement and modification by loxP-mediated evolution.

Although Boeke’s team wanted to make the genome unstable when they desired, they didn’t want the genome to undergo changes or rearrangements of its own accord, potentially disrupting the integrity of the synthetic strain. To increase the genome’s stability, they took out mobile DNA elements, such as retrotransposons, that in theory could jump to new spots at any time.

The design they pioneered with the chromosome 9 arm cuts out other noncoding DNA as well. The natural telomeres, the repetitive regions next to the ends of chromosomes that can also be unstable, are now gone. Shorter synthetic ones will cap each chromosome. The researchers also took out many of the introns, the noncoding sequences between coding regions of a gene.

The quest for stability also prompted some radical steps. The team is taking out the DNA coding for the yeast’s 275 transfer RNAs, which shuttle amino acids to the ribosome for stitching together into a protein. The transfer RNA genes are essential, but because their sequences can sometimes cause a cell’s DNA copying machinery to stall out, they are “DNA damage hotspots,” says Patrick Yizhi Cai of the University of Edinburgh in the United Kingdom. Cai is building a “neochromosome” that will have all those genes relocated onto

it. Collected into one place, these genes will still be available to the modified organism, but will do less damage if they become unstable. (The neochromosome will not increase the total number of chromosomes in Sc2.0, because Boeke plans to do away with chromosome 1. It naturally has just 230,000 bases, and given the design rules of Sc2.0, its synthetic counterpart would be as much as 70,000 bases shorter. Worried that this diminished chromosome would be unstable, the team plans to append that DNA to another synthetic chromosome.)

Boeke and his colleagues added two more modifications to the design. Throughout the genome, they are inserting short, specific DNA sequences, detectable by the polymerase chain reaction, that distinguish each synthetic chromosome segment from its natural counterpart. Finally, they tinkered with some of the natural stop signs in eukaryotic genomes—the “stop” codons that tell a cell when to cease making an RNA. On the chromosome 9 arm, the researchers turned every single instance of one stop codon, “TAG,” into another, “TAA,” by switching in the base adenine (A) for a guanine (G). In the complete synthetic genome, they will

make more than 1000 such substitutions all together. The “stop” sign is therefore still there, but in a different form. But this frees up “TAG” as a codon for an artificial amino acid, if they ever decide to introduce one into the makeup of their enhanced yeast.

With the aid of a software program developed by computer scientist Bader, Boeke designed a version of the chromosome 9 arm incorporating all of these changes and carefully checked as much as possible that the added bases would not interfere with the expression of any remaining yeast genes. He then contracted with a

biotech company called Codon Devices to synthesize the chromosomal arm.

Eleven months went by with no word from the company, which had never attempted to make such a long piece of DNA. “That was a tense time,” Boeke recalls.

But that dark period proved inspirational as well. Boeke wondered whether he could speed up and decrease the cost of the work, as well as help others learn molecular biology,



“You can put it as a kind of milestone, like the first human genome.”

—JEFF BOEKE,
NEW YORK UNIVERSITY

if he set up a course dedicated to building a synthetic yeast genome (see sidebar, p. 1429). He launched the idea as a summer school offering in 2007, even before he knew that the 9R synthetic arm he had ordered would work. Now, 6 years later, the thrice-weekly course at Hopkins is packed, even on Friday nights. “We were overwhelmed with interest” from biology, engineering, computer science, public health, and other majors, says Boeke, who, despite his move to New York, will keep the Hopkins course going with colleagues there.

Scrambled DNA

Once Codon Devices finally delivered a 90,000-base circular chromosome, it took Boeke and colleagues months more to successfully stick it into the yeast, cut out the natural 9R, and then test the effects. The synthetic chromosome arm performed without a hitch, yielding healthy yeast and reasonable gene expression, Boeke’s team reported in 2011 in *Nature*.

The SCRaMbLE system worked as well. The yeast carry a piece of DNA that codes for a highly modified version of an enzyme called Cre recombinase, which randomly deletes or inverts the DNA lying between any pair of loxP sites. This enzyme is usually stuck in an unfolded form in the cell’s cytoplasm, but when the researchers add the chemical estradiol to yeast, it folds up and can sneak into the nucleus. LoxP activation leads to the random removal of different genes, sometimes killing the yeast but other times simply changing the yeast’s properties, rendering it incapable of making certain amino acids, for example. “We knew our design had panned out,” Boeke says. “Our hope then was to scale up.”

Meanwhile, the students from that initial summer course and subsequent ones had taken on chromosome 3. From an initial class goal of 1500 bases per student,

productivity had ramped up the point where each student promised to turn in at least 30,000 bases by the semester’s end. It took 49 students and 1.5 years to make the synthetic chromosome’s 272,871 bases—the native version has 316,667 bases—but under the guidance of Boeke’s postdocs Narayana Annaluru and Héloïse Muller, their effort has paid off with a publication online this

week in *Science* (<http://scim.ag/NAnnaluru>). Yeast carrying the shortened, modified chromosome grew normally and looked little different from their natural counterparts under almost all the growing conditions tested, the researchers report.

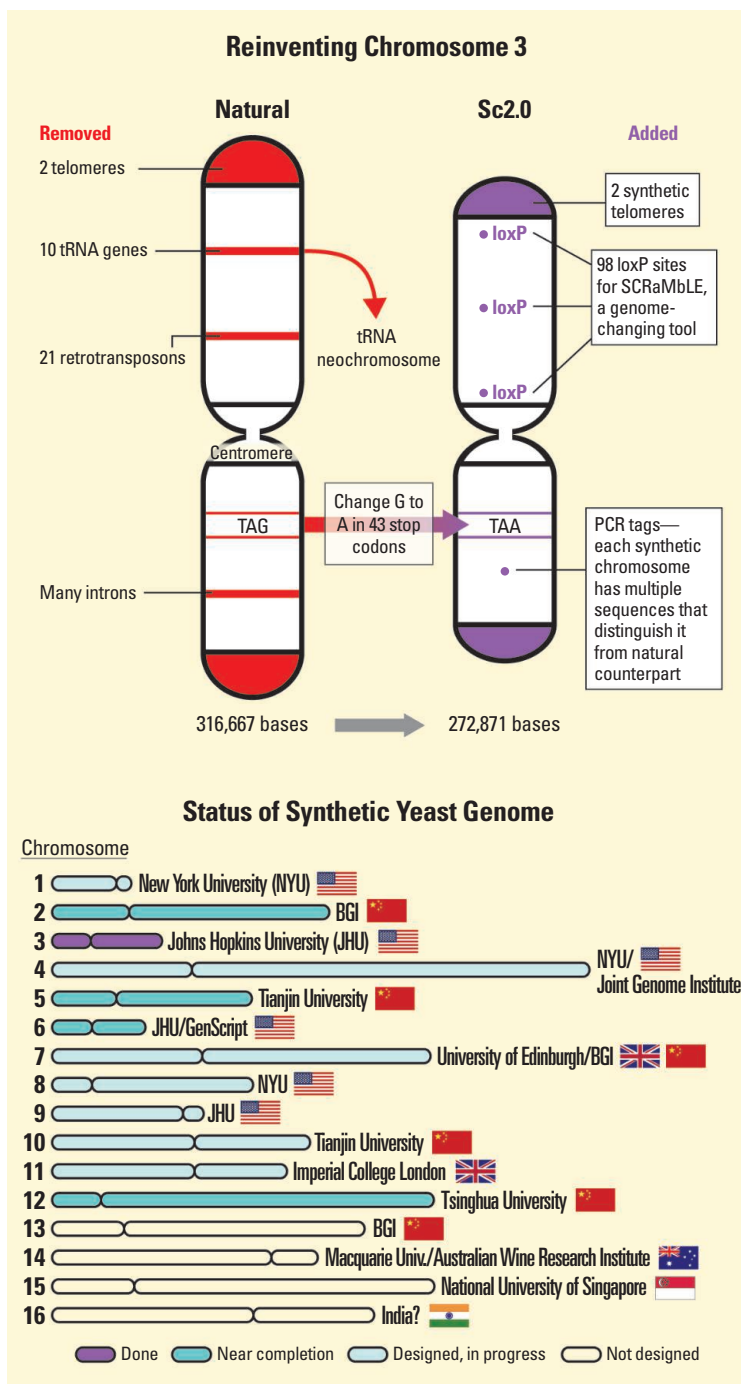
“They made some pretty dramatic changes,” says Mike Tyers, a yeast systems biologist at the University of Montreal in

Canada. “But on the other hand, they were conservative enough that the experiment would have a chance of working. I think they hit the sweet spot.”

As Boeke and his colleagues entered the home-stretch with chromosome 3 and began to work on other yeast chromosomes, a chance meeting between Cai, then Boeke’s postdoc, and Ying-Jin Yuan of Tianjin University in China at a synthetic biology competition led to the globalization of the effort. “An international project wasn’t on our mind at all,” Boeke recalls. Yet Yuan was so excited about Sc2.0 that he got Huanming Yang of the sequencing powerhouse BGI involved. Yang helped organize the first synthetic yeast genome meeting, held in Beijing in 2012, and partnerships emerged. Yuan set up his own “Build A Genome” course and in the summer of 2012, 60 Chinese students assembled all the bases needed for chromosome 5.

Momentum built elsewhere as well. Tom Ellis, a synthetic biologist at Imperial College London, became hooked on the ambitious venture after attending the Beijing meeting. He helped organize a second Sc2.0 meeting in July 2013, following a larger synthetic biology conference at which the British government announced it would commit £1 million toward the synthetic yeast genome project. Other countries are getting involved as well, Boeke says (see chart).

The hope is that in 2 years, the various partners will have stitched together each of the



Genome remake. The successful reinvention of yeast chromosome 3 involved the removal of many elements and multiple additions to its DNA (upper diagram). This chromosome now serves as a model for the international effort to synthesize all the other chromosomes (lower chart).

yeast's chromosomes, with strains containing one chromosome each. Then Boeke will tackle the difficult task of putting them all in one organism. "It's a little surprising that we can have this grand idea with so many moving parts" and multiple organizations making different chromosomes, says Boeke's senior lab coordinator, Katrina Caravelli. But so far, "it's working well."

Custom yeast

Once the synthetic organism is in hand, yeast biologists will be eager to tinker with it. With 5000 loxP sites, the synthetic genome will be ripe for mutations, opening the way for researchers to take a systems approach toward understanding which genes matter when during the yeast life cycle.

The longer they expose a yeast strain to estradiol, the greater the number of genetic changes that the SCRaMbLE system will induce. Leslie Mitchell, a postdoc in Boeke's lab, plans to grow the mutated strains under different conditions, document their behavior and other characteristics, and sequence their genomes. From the data, she hopes to piece together how different genes interact.

SCRaMbLE should also make it easier to harness yeast for biotechnology. Right now, finding a yeast strain that makes enough of a product to be commercially viable "takes many steps and many person years," Boeke says. SCRaMbLE should speed that search up by providing an "unbelievable number of mutants that would be much more time-consuming to get one at a time." With more strains to evaluate, researchers are more likely to find the best one for the job.

Success at synthesizing versions of yeast's native chromosomes will also open the way to adding entirely novel chromosomes to the organism—ones that endow it with brand-new properties or enable it to model human diseases. Boeke, for example, would like to install in yeast all of the genes of the molecular pathway that, in humans, is defective in Lesch-Nyhan syndrome, a rare disorder characterized by gout and kidney and neurological problems, including self-mutilating behavior. By modeling the molecular defect in yeast, he hopes to figure out how the mutation affects eukaryotic cells in general.

Researchers who have watched the steady progress made by Boeke's army say the project will be a boon for basic science, promising "deeper mechanistic understanding of biological processes in yeast," says Huimin Zhao, a synthetic biologist at the University of Illinois, Urbana-Champaign.

Student Assembly Drives Yeast Project

The homework that James Chuang and Katrina Caravelli turned in to Jef Boeke consisted of just four letters: A, C, G, and T, representing DNA's four bases, each repeated thousands of times. But there was nothing tedious about their assignment: "Build A Genome," as the undergraduate course's name put it. Boeke conceived the course at Johns Hopkins University in Baltimore, Maryland, 6 years ago while struggling to figure out how to do all the work required to build a synthetic yeast genome (see main story, p. 1426).

For Boeke, the course supplies labor—scores of undergraduates have taken it so far. For students, it offers intensive training in synthetic biology and the thrill of taking part in frontline research. "I was really fascinated by the potential and by my ability to have an impact," recalls Caravelli, who signed up for the course in 2008 and is now Boeke's senior lab coordinator at New York University. During the semester-long course, students learned basic molecular biology procedures such as performing polymerase chain reactions and cloning DNA in bacteria.

Each student committed to completing 10,000 DNA bases during the course. "My building blocks went to chromosome 8," Chuang, now a graduate student in biomedical engineering at Boston University, says proudly. After being supplied with the DNA sequence he needed to synthesize, he started out with 16 pieces of DNA about 75 bases long, ordered from a commercial DNA synthesis firm. The ends of some pieces overlapped, so when he mixed the pieces together with enzymes, they self-assembled into 750-base spans dubbed building blocks. After making sure the building blocks had the correct sequence, he put them into bacteria to generate many copies of the newly assembled DNA. Now, Johns Hopkins graduate student Jingchuan Luo is putting those bigger DNA sections into yeast and making "minichunks" about 3000 bases long. If the yeast stays healthy, then she adds the next chunk in line to it, and so on. Her goal: to make a yeast strain with a totally new chromosome 8.

Caravelli recalls that her own bacteria sometimes wouldn't grow with the yeast segment in their midst. She enjoyed figuring out why. "The trial-and-error process challenges you to think properly like a scientist."

Boeke's course has proved such a success that three other U.S. universities are hosting or will soon host their own. Because it's now cheaper to buy 750-base segments than to have students make them, current students start with such DNA blocks and turn them into 3000-base spans, and, ultimately, 10,000-base chunks. Class productivity has soared. Each student's target is now 30,000 to 50,000 bases.

The students have high hopes for their work. "I want to see synthetic biology do really useful things for society," Chuang says. "The synthetic yeast genome provides a template for doing [that]."

—E. P.



Genome builder.
Katrina Caravelli.

But Kirsten Benjamin, a synthetic biologist at Amyris Inc., the Emeryville, California, company that harnesses yeast for producing artemisinin and other chemicals, expects that problems loom for the project: The more synthetic DNA Boeke tries to incorporate in a yeast, the sicker the strain will likely be, she predicts. "We're going to find a bunch of ways where it doesn't work," she says. But she agrees that the problems could be revealing, saying they "will allow us as a scientific community to discover all these unappreciated phenomena."

She and others are not sure how useful synthetic genomes will be for commercial applications. Less drastic approaches to improving yeast's manufacturing prowess may work better. "From a practical viewpoint, it is too costly to [make synthetic genomes] for most engineering applications," Zhao says.

But maybe it's enough to just build a eukaryotic genome once, Boeke suggests. "In a way, you can put it as a kind of milestone, like the first human genome was a milestone for genomics," he says. "When we finish it, we can really plant a flag in it."

—ELIZABETH PENNISI

LETTERS

edited by Jennifer Sills

Australia's Drought: Lessons for California

MOST OF CALIFORNIA IS SUFFERING FROM AN extreme drought, and storage levels in the major reservoirs are well below historic levels. For the past several months, an unusually stubborn ridge of high pressure off the West Coast of the United States has been blocking normal winter storms and the rain they carry. California's history of drought has led to state-wide strategies to save water, but Californian residents and policy-makers can do even more: They can look to the story of Australia's experience with a drought so intense and long-lasting that it was dramatically dubbed the Millennium Drought (1).

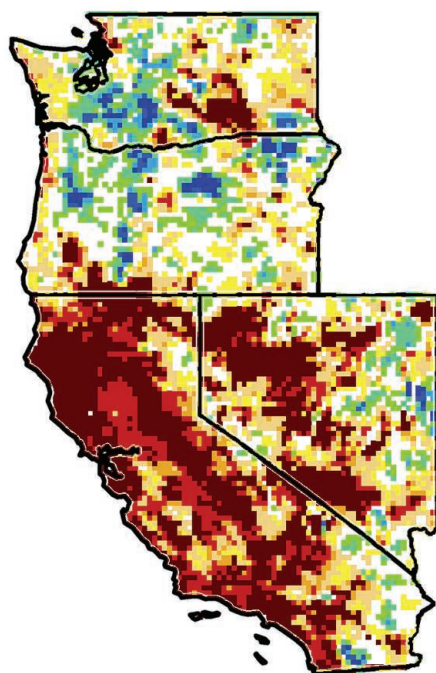
The Millennium Drought lasted from 1997 until late 2009 (2). Australia's economy and environment were hit hard. The drought accelerated the same trends facing farmers in developing countries worldwide: Small farms were squeezed out. Midsized farms were most vulnerable because they could neither achieve the economies of scale available to larger producers nor buffer losses with off-farm employment like the smallest farms could.

Amazingly, despite blows to crop yields and livestock numbers, Australia's rate of growth in agricultural production has quickly returned to predrought trends. The impacts of this major drought on irrigation communities were buffered by some critical water reforms. These included: (i) well-developed water markets that allowed water trade to farmers in the greatest need; (ii) modernization of irrigation infrastructure that increased the efficiency of water delivery; and (iii) establishment of clear water entitlements for the environment that protected critical refuge habitats and populations as water availability declined.

The use of water markets was particularly critical. More than 40% of annual water allocations were traded at the height of the drought in 2007. For example, increased water prices allowed dairy farmers to sell their allocation and purchase fodder with the proceeds rather than irrigate pasture. Fruit growers and other producers who needed to maintain irrigation throughout the drought could purchase the dairy farmers' water to keep their operations viable.

In urban areas, strategies to increase supply and decrease demand were brought to bear. Expensive desalination and water recycling plants were built. Australians were more comfortable with the desalinated water (3, 4), despite the recycled water's safety and the desalination plants' greater cost and large carbon and environmental footprints (4).

Between 2002 and 2009, per capita municipal water use in southeast Australia decreased by nearly 50% (5). Water use restrictions ranged from outright bans of conspicuously con-



Dried out. As of February 2014, most of California is in Extreme to Exceptional Drought (see red and dark red areas on map).

sumptive activities—such as daytime lawn watering and car washing—to rules promoting efficient water use—such as requirements for shutoff valves on hoses. Out of those temporary restrictions, permanent restrictions grew. Some areas in Australia still restrict daytime sprinkler use. Perhaps most relevant for worried Californians is how the Australian public received these changes. Studies cite an overall spirit of goodwill and cooperation fostered by the stress of drought (6).

The Millennium Drought brought about profound changes in Australians' conception of the environment, climate change, and water. The sticking power of those lessons and the success of the resulting policies and strategies will be tested by the next big drought. One lesson California can glean from the Australian experience is empowerment. Individuals making frugal water decisions can make a big difference in urban areas. Water markets and other measures that increase the flexibility of irrigation farmers in their response to drought can have big payoffs. Sustaining critical environmental water requirements will provide the basis for postdrought environmental recovery. A spirit of cooperation rather than contention can prevail even when tough decisions are made to address the needs of farmers and city residents.

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Drug Trafficking's Effects on Coastal Ecosystems

IN THEIR POLICY FORUM "DRUG POLICY AS conservation policy: Narco-deforestation" (31 January, p. 489), K. McSweeney *et al.* explain how Central American drug trafficking contributes to forest loss. Coastal ecosystems are also affected by drug trafficking.

Coastal drug trafficking is prominent along the Mesoamerican corridor (1). Shrimp and lobster fishing ports often double as trafficking centers, and small reef islands are occupied by narco-traffickers

whose clandestine operations seed fear and corruption (2, 3). Fishers get pulled into the drug trade, as they can make small fortunes selling supplies to traffickers, working directly with them, or by hunting the "white lobster"—bales of cocaine cast adrift by traffickers dumping evidence when capture is imminent (2–4). Drug traffickers also invest heavily in fishing fleets to help hide their operations, a narco-capitalization that could facilitate overfishing of high-value species such as shrimp or lobster (4–7). Money laundering also endangers coastal ecosystems through the development of often unregulated projects (e.g., building hotels) (8). The effect of these illegal activities on fisheries and coastal management is unquantified. As we study how "drug policy is conservation policy," let's not forget the coast.

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Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to www.submit2science.org.



theBUZZ

Keystone XL

In her 21 February Editorial, "Keystone XL" (p. 815), Marcia McNutt endorses the Keystone pipeline, provided that the Canadians reduce greenhouse gas emissions (GHGs) from the extraction and production process, that a tax is imposed on the pipeline to make fossil fuels more expensive and support renewable energy, and that a successful review of the pipeline's environmental impact statement is completed. Many readers responded to her proposal. Excerpts from those comments are below. See all comments at <http://comments.sciencemag.org/content/10.1126/science.1251932>.

A selection of your thoughts:

...I think one of the key impacts of development of the Canadian tar sands that goes uncovered in the American media is on the lives, culture, and land of indigenous First Peoples in Canada [We] need to curtail tar sands development by whatever means the United States can marshal: economic, legal, diplomatic/political, environmental, and moral.

—Josh Foster

Poor argument. If this pipeline wasn't important to the further development of the tar sands, it's unlikely that the Canadian government would be putting so much effort into lobbying for the pipeline. ...There should be better ways of mitigating climate change than blocking this pipeline,

but the inability of our political system to make sensible policy in this domain leaves few alternatives. The idea that the Obama administration can use the Keystone pipeline to get the Canadian government to behave more responsibly is a delusion. There can be no responsible development of the tar sands....

—Roger Albin

...If readers of *Science* want to advocate conditions for allowing the pipeline to move forward, I suggest the following: Enact a carbon price that rises year-by-year to reach \$50 or more per ton of carbon by 2030. Make it clear to investors that the carbon price will have to be paid for all the oil that moves through any pipeline, and that the pipelines must meet other criteria for environmental protection. Then let the market do what it will. But so long as we give fossil energy extractors a free pass to dump GHGs into the atmosphere with no constraint, we should hold the line on projects such as the XL Pipeline.

—Neil Leary

...I have yet to read how any of the opponents of the Keystone pipeline explain how eliminating it will have any impact at all on a bad Canadian decision and why the pipeline is worse than the methods that will be used to transport it—i.e., rail and truck. Perhaps canceling the pipeline might be some sort of object lesson, but this seems to me to be more in the feel-good category than a constructive one.

—Peter Geiser

Peter,... The symbolism of what we do can have profound impacts unrelated to the magnitude or even the direction of our actions. I'm particularly concerned if the symbolic consequence of our actions tips the balance unfavorably in other nations on the brink of making critical climate change decisions.

—Fred Moolten

HISTORY OF SCIENCE

A Hardbound Information Revolution

Bernard Lightman

James Secord's previous book, *Victorian Sensation (I)*, has become highly influential due to its pioneering integration of scholarship in the field of print culture with the history of Victorian science. In that book, Secord (a historian of science at the University of Cambridge) explored the communications revolution that took place in Britain during the second quarter of the 19th century, spurred on by new developments in print technology, which led to a vastly increased reading public. He argued that the resulting changes in both the reading and publishing of science had a profound effect on the very nature of science.

Secord built *Victorian Sensation* around an exhaustive study of Robert Chambers's *Vestiges of the Natural History of Creation* (1844). He shows how *Vestiges* captured the imagination of its many readers (about 39,000 copies had been sold by 1890) with a dramatic evolutionary epic, from monad to human. One of the many strengths of *Victorian Sensation* is Secord's analysis of the reactions of a range of readers to their encounters with *Vestiges*. Drawing on a multitude of different types of sources, including diaries, journals, and correspondence, Secord detects important patterns in the way *Vestiges*'s large readership interpreted the book. But despite its success, *Victorian Sensation* raised some questions about the feasibility of applying insights from the field of print culture to scientific texts: How many 19th-century books were, like *Vestiges*, read widely by readers whose reactions have been preserved in a form that is accessible to historians? In other words, are there any science books from this period, other than Charles Darwin's *Origin of Species* (1859), that could be analyzed using Secord's approach?

In *Visions of Science*, Secord offers answers to these questions by showing how print culture considerations illuminate the importance of seven key scien-

tific texts from around 1830: Humphry Davy's *Consolations in Travel* (1830), Charles Babbage's *Reflections on the Decline of Science in England* (1830), John Herschel's *Preliminary Discourse on the Study of Natural Philosophy* (1830), Mary Somerville's *On the Connexion of the Physical Sciences* (1834), Charles Lyell's *Principles of Geology* (1830–1833), George Combe's *Constitution of Man* (1828; republished 1835), and Thomas Carlyle's *Sartor Resartus* (first published in serial form 1833–1834). It is not immediately obvious what ties these seven works together. But for Secord they belong to a new genre of scientific writing that he refers to as “reflective scientific treatises.” They were literary experiments. Characterized by a focus on the underlying methods and principles of science and its place in modern life, the books also presented imaginative visions of the future. It is no coincidence that they appeared around the same time, a period of unparalleled change. The books offered “comprehensive perspectives on science and its meaning for human life, at a moment when [Britain] appeared on the brink of revolution” and when “the country confronted the prospect of a greatly expanded reading public.” Each chapter of *Visions of Science* explores

the making and reception of one of the books “as a focus for public controversy.”

Throughout, Secord draws continuously on methods developed by print culture scholars. The physicality of the books is important. For example, Secord points out that Somerville's *Connexion*, which used a small octavo format, delicate type, and generous spacing, was typical of the reflective works on science published by John Murray that attempted to address a wide range of readers. Secord considers prices and sales of each of the seven titles. In the case of Combe's *Constitution of Man*, he discusses how the republication of the book by the Chambers publishing house in a much cheaper form dramatically increased the readership. By reducing production costs to a bare minimum and exploiting the spare capacity of the firm's steam printing machine, the book could be sold for one shilling and six pence. As a result,

it became one of the best-selling books of the 19th century. Secord sees the sales of *Constitution* as “the culminating point of the effects of the new technologies of print and its distribution in the first half of the nineteenth century.” In another demonstration of the importance of understanding the publication history, Secord discusses how Lyell's *Principles of Geology* was repackaged for a wider market only after the first two editions had appeared. According to Secord, this was a deliberate strategy that reflected Lyell's belief in an elitist, authoritarian vision of “popular science.”

When it comes to analyzing the reactions of readers, Secord lacks the wealth of primary sources that he drew on for his earlier study of *Vestiges*. He must rely more on periodical reviews than on letters or diaries. Nevertheless, his discussion of the reception of these works is always revealing. His examination of the reviews of Somerville's *Connexion*, for example, shows that it was positively received because readers understood that it was not a primer but rather an attempt to draw attention to the connecting links between different scientific disciplines.

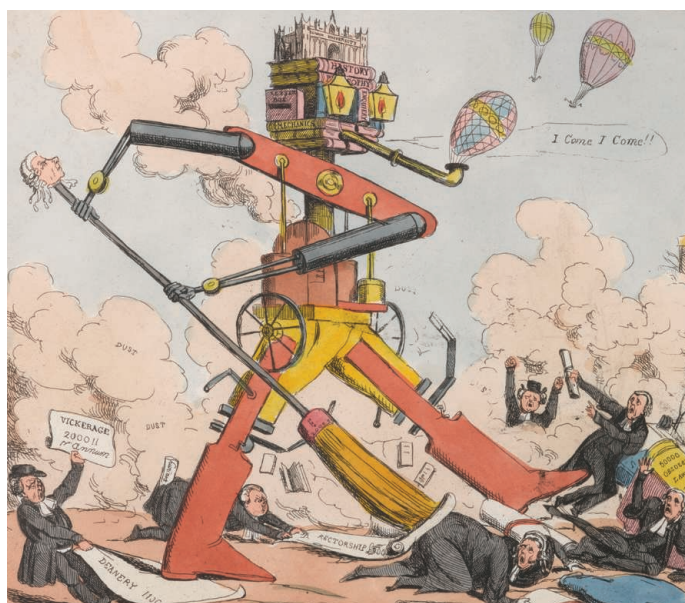
One finds the payoff of the print culture perspective in the fresh interpretations of each text that Secord gives us. Herschel's *Preliminary Discourse*, usually read as a treatise on induction,

Visions of Science

Books and Readers at the Dawn of the Victorian Age

by James A. Secord

Oxford University Press,
Oxford, 2014. 328 pp. £18.99.
ISBN 9780199675265.



The March of Intellect (detail). Colored etching by Shortshanks (Robert Seymour), published by T. McLean, London, 1829.

is actually a conduct manual for scientists. Herschel wanted to encourage the qualities of moral probity, truth-telling, and religious faith. He aimed to provide a model for the actions of the ideal seeker after truth. Here, context is all. Herschel knew that his work would be read by contemporaries who were experiencing the changes sweeping through the publishing world that were affecting all of British society. His book was grounded on the assumption that reading had the power to transform the human condition. In his *Principles of Geology*, Lyell was not just trying to establish geology on a secure foundation. He was also attempting to help his readers come to terms with the consequences of scientific discoveries in relation to the biblical accounts of the Creation and the Flood. Lyell had to write about geology in a way that would calm fears of materialism and irreligion.

Elegantly written, Secord's *Visions of Science* provides its readers with fresh insights into the turbulent decade around 1830, when science was changing from a "relatively esoteric pursuit" into one that would have a huge impact on "the everyday life of all men and women."

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10.1126/science.1251427

PHYSICS

Tracking Subatomic Physicists

Matthew Reece

The documentary *Particle Fever* resolutely places scientists, more than science, in its focus. Director Mark Levinson has a physics background; producer David E. Kaplan is a theoretical physicist who was once a film student. They saw in CERN's Large Hadron Collider (LHC) an extraordinary opportunity to capture a major development in science as it was happening. As Kaplan says, "I've never heard of a moment like this in history, where an entire field is hinging on a single event."

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And the event is massive: The LHC hosts four collaborations, those around the biggest two particle detectors (ATLAS and CMS) comprising several thousand physicists who share credit for discovering the Higgs boson. *Particle Fever* makes their epic undertaking accessible by acquainting us with a few theorists (Savas Dimopoulos, Nima Arkani-Hamed, and Kaplan) and ATLAS experimentalists (Monica Dunford, Fabiola Gianotti, Martin Aleksa, and Mike Lamont). We see them at ordinary, humanizing moments: Dunford bicycling near Geneva, Gianotti playing the piano, Arkani-Hamed and Kaplan playing ping-pong. They are our guides not only to the science of the LHC but also to the emotional trajectory of those taking part in it. "The entire control room is like a group of six-year-olds whose birthday is next week," Dunford explains shortly before the first proton beams circulate in the LHC, and her own excitement at the first data is irrepressible. Ruminating on how data have begun to falsify some of his ideas, Dimopoulos explains that for a theorist "jumping from failure to failure with undiminished enthusiasm is the big secret to success."

Much of the film centers on the search for the Higgs boson. In an interview with the *Guardian*, the film's editor, Walter Murch, noted that "the Higgs boson is kind of a MacGuffin," the Hitchcockian plot device that motivates the characters but remains opaque to the audience (1). The film eschews the elaborate analogies characteristic of much popular science exposition, with Kaplan explaining simply that "[the Higgs] gives particles like the electron mass. ... Without the Higgs, life as we know it wouldn't exist." Rather than trying to convince the audience that the Higgs matters, the film shows that it does by following people who are intensely invested in the experiment's outcome.

Although the film does not concentrate on the exposition of scientific details, it gets them right except for minor oversimplifications. Early hints of a 140-GeV Higgs mass in 2011 are said to favor multiverse theories as opposed to supersymmetry, whereas that prediction actually relied on supersymmetry in a crucial way—just at a higher energy than in conventional theories. The film nonetheless accurately conveys theorists' confusion about



Dwarfed by detector's face. Gianotti and Kaplan in the ATLAS cavern.

how to interpret the measured mass of 125 GeV, which ruled out many favorite theories.

Running through *Particle Fever* one finds a sustained, if sometimes implicit, argument that curiosity-driven science like the LHC should be viewed in common with great art as a treasured cultural achievement of humanity. Gianotti explains that as a young person she studied art, literature, and music. She draws an analogy between musical harmony

and the scientific laws she seeks to uncover now. In one skillfully edited scene, a tense crowd waits in the ATLAS control room on 30 March 2010. At the moment the first high-energy collisions begin, displays trace the paths of particles through the detector, the crowd erupts in cheers, and the soundtrack blasts Beethoven's "Ode to Joy." We see a succession of images from centuries of art and science, including a play on the visual similarity of ATLAS event displays and rose windows. The ATLAS detector itself, described by Dunford as "a five-story Swiss watch," is a visual star of the film, every bit as large and intricate as a Gothic cathedral and making a similar statement about the vision, skill, and shared goals of the society that built it.

Near the end of *Particle Fever*, Arkani-Hamed extols Werner Herzog's documentary *Cave of Forgotten Dreams* [reviewed in (2)], about Paleolithic art. Kaplan draws out the comparison, characterizing scientists, like early explorers, as people "going into the frontier" and painting amazing pictures of what they found. *Particle Fever* paints such a picture by packaging the LHC's exploratory science in the form of a compelling, personality-driven narrative.

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10.1126/science.1251659

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Next Steps for Citizen Science

Strategic investments and coordination are needed for citizen science to reach its full potential.

Rick Bonney,^{1†} Jennifer L. Shirk,¹ Tina B. Phillips,¹ Andrea Wiggins,^{2,1} Heidi L. Ballard,³ Abraham J. Miller-Rushing,^{4*} Julia K. Parrish⁵

Around the globe, thousands of research projects are engaging millions of individuals—many of whom are not trained as scientists—in collecting, categorizing, transcribing, or analyzing scientific data. These projects, known as citizen science, cover a breadth of topics from microbiomes to native bees to water quality to galaxies. Most projects obtain or manage scientific information at scales or resolutions unattainable by individual researchers or research teams, whether enrolling thousands of individuals collecting data across several continents, enlisting small armies of participants in categorizing vast quantities of online data, or organizing small groups of volunteers to tackle local problems.

Despite the wealth of information emerging from citizen science projects, the practice is not universally accepted as a valid method of scientific investigation. Scientific papers presenting volunteer-collected data sometimes have trouble getting reviewed and are often placed in outreach sections of journals or education tracks of scientific meetings. At the same time, opportunities to use citizen science to achieve positive outcomes for science and society are going unrealized. Here, we offer suggestions for strategic thinking by citizen science practitioners and their scientific peers—and for tactical investment by private funders and government agencies—to help the field reach its full potential.

Transformed by Technology

Although citizen science is sometimes considered a recent phenomenon, amateur scientists have studied the world for most of recorded history (1). Much of our current understanding about our natural environment, including the effects of climate change, is derived from data that have been collected, transcribed, or processed by members of the public. During the past two decades, the number of citizen



Training for data-gathering. Women from Komo (Republic of the Congo) learning to map in the forest, as part of the Extreme Citizen Science (ExCiteS) Intelligent Maps project.

science projects, along with scientific reports and peer-reviewed articles resulting from their data, has expanded tremendously.

Much of this growth results from integration of the Internet into everyday life, which has substantially increased project visibility, functionality, and accessibility. People who are passionate about a subject can quickly locate a relevant citizen science project, follow its instructions, submit data directly to online databases, and join a community of peers. eBird, for example, engages the global bird-watching community to collect more than five million bird observations every month and to submit them to a central database where they can be analyzed to document the abundance and distribution of bird populations.

The Internet also has enabled citizen science projects that can be accomplished only online. Many are data-processing projects for which participants classify or interpret sound files, videos, or pictures, such as the millions of images of galaxies, moon craters, and sea-floor organisms that have been categorized by participants in various projects operated through Zooniverse.

Citizen science also has been enhanced by statistical tools and computational techniques that remove many of the barriers to compiling and analyzing complex data sets. Computers and accessible interfaces have made participa-

tion possible for groups that previously were not reached or well served by citizen science, such as those with literacy or numeracy skills that are not text based (2).

Scientific Impact

Some people question the practice of citizen science citing concerns about data quality. With appropriate protocols, training, and oversight, volunteers can collect data of quality equal to those collected by experts (3). For large projects where training volunteers and assessing their skills can be challenging, new statistical and high-performance computing tools have addressed data-quality issues such as sampling bias, detection, measurement error, identification, and spatial clustering (4, 5).

As an illustration of data quality, data from eBird have been used in at least 90 peer-reviewed articles and book chapters covering topics in ornithology, ecology, climate change, and statistical modeling (6). Zooniverse projects have yielded more than 50 peer-reviewed articles on topics ranging from galaxies to oceans (7). And many environmental protection agencies use volunteer water- and air-quality data to target streams and neighborhoods for protection.

Understanding the scientific impact of citizen science can be challenging because of the spectrum of projects that are referred to

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by that name. As the field matures, we hope that the term “citizen science” will be used to describe projects, regardless of size, that truly do science—that produce reliable data and information usable by anyone, including scientists, policy-makers, and the public, and that are open to the same system of peer-review that applies to conventional science. To ensure that critiques of citizen science efforts are based on merits of the research rather than unfounded assumptions about the practice, project developers must employ sound research or monitoring design, and reviewers should look for evidence of such practices in their appraisals (8).

Those who seek to build capacity in the citizen science field can help by developing and improving open-source data management technologies, data analysis tools, professional development opportunities, and project evaluation services similar to those already available in other fields of science. These services would help to address skepticism about data quality. Many guides, tools, and templates are available to support citizen science project planning, testing, maintenance, and evaluation; formal data policies; and data management and quality-control plans (e.g., citizenscience.org, citizensciencealliance.org, and citsci.org).

Social and Environmental Impact

Although citizen science projects should have authentic scientific objectives, they also can realize significant social outcomes. The sea turtle monitoring network Grupo Tortuguero supports a body of hypothesis-driven scientific work, including investigations into turtle diet, distribution, and disease, at sites throughout northwestern Mexico (9). This collaboration between biologists, agencies, and communities has helped to establish marine protected areas and sustainable fisheries practices that are sensitive to the well-being of both turtle populations and local livelihoods. The West Oakland Environmental Indicators Project empowered individuals living in a very poor neighborhood to collect air-quality and health data documenting the degree to which air pollution affects local residents (10). And in the Congo, scientists from University College London are leveraging the data-capture capabilities of smartphones to work with nonliterate individuals to document environmental impacts, such as poaching and illegal logging (2) (see the photo).

These examples demonstrate how citizen science can provide opportunities for people of many backgrounds and cultures to use science to address community-driven questions. Creating projects to achieve social and sci-

entific objectives requires deliberate design that is attentive to diverse interests, including why and how members of the public would even want to be involved (11). Investments in infrastructure and partnerships that help to create more local projects with both science and social components could leverage underappreciated knowledge sources, including local and traditional knowledge. Such efforts could also inform the questions and issues pursued through citizen science, leading to new research and a stronger science-society relationship.

Organizing to Maximize Impact

The growing number of citizen science projects around the world is inspiring. On the other hand, a lot of new projects are not really “new.” Many different projects collect similar data in similar locations, which confuses the pool of potential participants and results in numerous patchy data sets rather than a few large and truly useful ones. One solution to reduce project redundancy is for scientists and project developers to adopt, adapt, or collaborate with already-proven projects and to fit them to their area or topic of interest. This approach also reduces the expense of design, testing, and implementation when a project is started “from scratch.” eBird, for example, has developed “portals” that allow partners to customize the program for specific regions or data-collection processes, while still channeling all program data into one accessible repository.

Project developers could also look for opportunities to gather truly important information in ways that are currently going unrealized. For example, citizen science could play a stronger role when natural or human-caused disasters or other unique data-collection opportunities occur. In 2009, the Jamaican Water Resource Authority required data on water levels from remote sites that could not be monitored readily by automatic equipment. The authority enlisted and trained volunteers to read river gauges at assigned locations, gathering data needed to implement protective measures before floods (12). As another example, the Famine Early Warnings Systems Network enlists local monitors to report data, such as rainfall and staple food prices, around the world for use in ensuring food security (13). Many existing citizen science projects could be enhanced by preparing protocols and volunteer infrastructure to enable scientifically sound data collection during and after recurring disaster situations (e.g., oil spills, wildfires, or earthquakes).

To help facilitate development, organization, and innovation of the field, a consortium

of individuals and organizations has created an international Citizen Science Association (CSA; www.citizenscienceassociation.org). The group’s goals are to promote and support citizen science best practices in such areas as data management, scientific rigor, ethics, and project evaluation. The CSA is also working closely with regional organizations such as the newly formed European Citizen Science Association. Input from a wide range of scientists and educators and investment in the CSA’s infrastructure by a variety of funding agencies would meet a critical need.

Going beyond the networking and tool development activities of the CSA, citizen science projects could be coordinated around the world to synthesize and analyze their diverse data sets to better understand significant scientific and socially relevant issues such as climate change. Most citizen science projects work independently, and many citizen science data sets containing a wealth of information are unknown or unavailable to decision-makers. The process of making these data accessible and usable as a tool for meeting some of science and society’s grand challenges would be facilitated by organizing around citizen science centers.

Centers for citizen science could create, organize, and synthesize centralized repositories of volunteer-collected data on topics such as water quality, phenology, biodiversity, astronomy, precipitation, and human health. Centers also could help to coordinate questions being asked of citizen science data, methods of answering those questions, and techniques for achieving educational and community-development goals for participants. As such, centers for citizen science would be excellent strategic investments for both private and government foundations.

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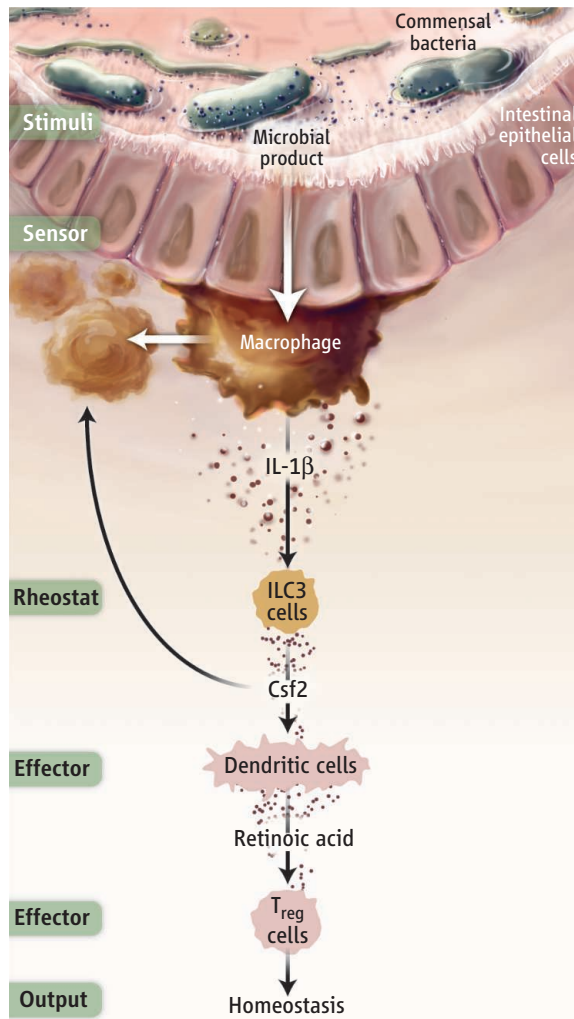
IMMUNOLOGY

The Axis of Tolerance

Tegest Aychek and Steffen Jung

The gut poses a unique challenge to the organism. The host must tolerate beneficial commensal bacteria, yet allow for rapid protective responses to invading pathogens. Failure to maintain this balance may cause intestinal disorders such as chronic inflammatory bowel disease. Fortunately, a dynamic, yet robust, state of chronic, low-grade inflammation maintains this balance (“primed homeostasis”). Thus, the host actively engages the gut microbiota, controlling its composition by secreting antimicrobial peptides and immunoglobulins. Conversely, commensals shape the gut-associated immune system by controlling the prevalence of distinct T cell populations (1). On page 1477 of this issue, Mortha *et al.* (2) show how communication between specific myeloid and lymphoid cells controls immune homeostasis under exposure to the gut microflora. Such cellular details of this steady state may guide the development of rational therapies for intestinal inflammatory conditions.

Intestinal macrophages are strategically positioned below the intestinal epithelium and highly abundant in the lamina propria (3), where they engulf cellular debris. Whereas most other tissue macrophages are established prenatally and maintained throughout adulthood by longevity and limited self-renewal (4, 5), these cells display—in keeping with their rapidly changing surroundings and tonic mild inflammation—a high steady-state turnover in the gut. Intestinal macrophages are continuously renewed from monocytes that are recruited from blood and differentiate in the healthy gut into noninflammatory cells (6). Timely conditioning of monocytes to adopt specific gene expression signatures allows them to help maintain local tissue homeostasis. Intestinal dendritic cells (DCs), by contrast, can efficiently migrate to lymph nodes, thereby providing a critical link to adaptive T cell immunity (7). Gut DCs govern the intestinal prevalence of T helper 17 (T_H17), T_H1, and T regulatory (T_{reg}) cells,



and by this means, determine host resistance to pathogen challenges. More recently, innate lymphoid cells (ILCs) have been shown to play a key role in gut homeostasis. These cells lack antigen receptors and their activities are triggered by cytokines derived from neighboring cells. Gut ILCs promote the formation of isolated lymphoid follicles, structures that support “homeostatic” immune responses. A subset of ILCs [in which the transcription factor retinoic acid receptor–related orphan receptor gamma t (RORγt) functions in development] called RORγt-dependent group 3 innate lymphoid cells (ILC3s), maintains the intestinal epithelial barrier by secreting the cytokine interleukin-22 (IL-22), which has an important role in protecting the intestinal epithelium following injury or infection by pathogens.

Communication between different immune cells of the intestinal mucosa acts as a rheostat that maintains tolerance to gut microbes.

Steady-state communication. Upon sensing microbial products in the gut, macrophages secrete IL-1β, which triggers innate lymphoid cells (ILC3s) to produce Csf2. Csf2-exposed DCs release retinoic acid, which promotes the generation of T_{reg}. Csf2 also increases the number of macrophages and induces them to release IL-10, which might contribute to local effector T cell maintenance.

Mortha *et al.* identify an intriguing microbiota-triggered steady-state cross-talk between intestinal mononuclear phagocytes (macrophages and DCs) and ILCs that act as a rheostat for gut mucosal adaptive immunity. The core of this cellular circuit consists of macrophages acting as microbiota sensors, and ILC3s that modulate the size and functionality of the DC compartment by secreting colony-stimulating factor 2 (Csf2; also called granulocyte-macrophage colony-stimulating factor). Csf2 stimulates the growth and differentiation of mononuclear phagocytes. The impact on DCs ultimately affects adaptive T cell immune responses (see the figure). The authors observed that Csf2 production by ILC3s was impaired in mice whose macrophages were either transiently depleted or rendered unresponsive to microbial stimuli. Importantly, Csf2 production in these animals could be restored by exogenous IL-1β, suggesting that this “proinflammatory” cytokine is key to the macrophage–ILC crosstalk. Indeed, ILC3s of

mice lacking the receptor for IL-1β failed to produce Csf2. Thus, microbe-sensing, intestinal steady-state macrophages secrete IL-1β to induce the release of Csf2 by ILC3s, highlighting the recruitment of proinflammatory factors for maintaining gut homeostasis.

Unlike the growth-promoting activity of Csf2 on cultured cells, its functions in animals have long remained enigmatic. Csf2 is a key effector molecule produced by T_H17 cells that sustains inflammation induced by experimental autoimmune encephalomyelitis (8, 9). Unexpectedly, however, Csf2 is dispensable for the development of proinflammatory DCs derived from monocytes (10). Rather, Csf2 promotes nonlymphoid tissue DC homeostasis (10). The finding that ILC3s are a key steady-state source of Csf2 in low-

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grade “physiological inflammation” in the gut adds an intriguing new twist to this puzzle. Moreover, Mortha *et al.* show that Csf2 controls not only the amount of intestinal DCs, but also their functions. Specifically, DCs, as well as macrophages, isolated from mice lacking Csf2 displayed impaired expression of retinaldehyde dehydrogenase, the enzyme that generates retinoic acid. As a consequence, these DCs failed to support the generation of T_{reg} in ex vivo cultures—a defect that could be restored by addition of Csf2. The functional deficiency of these cells also translated into a general reduction in the number of colonic T_{reg} cells and an impaired in vivo generation of T_{reg} in a mucosal tolerance paradigm (oral tolerance to dietary antigens), both in mice deficient in Csf2 production as well as in animals specifically lacking ILC3s that produce Csf2. These findings establish Csf2 production by ILC3 as a critical steady-state rheostat for adjusting intestinal T cell immunity to the microbiota status. Notably, retinoic acid is also critical for the imprinting of gut homing of effector T cells and thus represents a central factor in gut homeostasis.

Csf2-producing ILC3s are concentrated in isolated lymphoid follicles, but it is unclear whether these structures provide a unique permissive environment or if the macrophage–ILC–DC axis operates throughout the lamina propria. Mortha *et al.* show that antibiotic treatment of mice reduces Csf2 production by ILC3s. Although this treatment reduces the commensal load, it also creates profound dysbiosis. Do specific bacterial species activate the macrophages, and might this explain why these cells are equipped with transepithelial dendrites to sense the gut lumen (11)? With macrophage sensors and DCs that relay information to T cells, Mortha *et al.* emphasize the dual role of mononuclear phagocytes in gut homeostasis. However, differential functions of macrophages and DC subsets in this context remain incompletely understood. Of note, special activities of these cells are probably overridden in cell cultures, which neglect restraints imposed by discrete location and an in vivo requirement for migration.

The findings of Mortha *et al.*, if translatable into the human setting, could have

major implications in the clinic. Impairments of Csf2 signaling, either due to ligand neutralization by autoantibodies or receptor mutations, have been associated with deteriorating intestinal bowel disease. However, clinical trials of this disorder with Csf2, performed under the assumption that it boosts immunity, so far have failed to yield conclusive benefit. The study of Mortha *et al.* suggests a more complex role of Csf2 in the pathology of this condition. Success of Csf2 therapy in inflammatory bowel disease will, hence, likely depend on the right choice of patients and personalization of treatments.

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ASTRONOMY

A Different Class of Planets

Alexis Brandeker

Everywhere we look, it seems, we find exoplanets—planets orbiting stars other than the Sun. It is now estimated that, on average, there is at least one planet for every solar-type star (1). The vast majority (>98%) of known exoplanets have been found not through direct imaging, but by careful observation of how the host star is influenced by the presence of a planet; whether by induced motion from gravity or a periodic occultation of the stellar light. These indirect methods are heavily biased toward finding planets near their star, as those are the planets that influence the star the most. On page 1490 of this issue, Dent *et al.* (2) present results that implicate a planet far out from its star, through a technique that links the location of CO gas in the disk around a young star to the influence of an unseen planet.

Disk structures induced by planets are not limited to regions near the star, so even planets in wide orbits around their star are detect-

able in this way. The challenge is that disks are faint, generally difficult to resolve, and are common only around young stars. It is thus not surprising that the system that Dent *et al.* choose to study is β Pictoris (β Pic), a young (~20 million years old), nearby (~20 pc) star harboring one of the brightest and largest disks known; indeed, the disk around β Pic was the first ever to be imaged (3), an occasion that this year marks its 30th anniversary. Moreover, from previous observations, a massive super-Jupiter called β Pic b is known to orbit the star at ~10 astronomical units (AU) (1 AU is the distance between Earth and the Sun). The planet was first inferred from its gravitational influence, by the massive warp of the inner disk that was observed in light scattered off dust grains (4). It took more than a decade before β Pic b was directly imaged and seen to orbit the star (5).

Dent *et al.* observe a clump of CO at 85 AU from the star. Because CO does not survive for long in the ultraviolet field from neighboring stars, the implication is that the CO must be currently produced, perhaps by the destruction of icy bodies through enhanced colli-

Detection of a carbon dioxide gas cloud suggests the presence of an exoplanet further out from its host star than usual.

sions in this particular region. The enhanced rate of collisions could be due to either a Neptune-sized or larger planet in a wide, 60-AU orbit (twice the orbital size of “our” Neptune), or a relatively recent (<0.5 million years) collision between Mars-sized objects at 85 AU. To learn which of these cases is correct, we simply have to wait and reobserve the system in a few years, as the scenario with the more massive planet is predicted to change the visible disk structure, whereas a structure induced by a Mars-sized collision will keep the disk in a short-term steady state. In either case, planets are found to be located further out than would be expected from a standard planet-formation scenario (6). This raises the same questions as arise after the unexpected discoveries of hot Jupiters (7) (Jupiter-mass planets with orbital periods of days) and cold Jupiters (8) (Jupiter-mass planets located tens to hundreds of astronomical units away from their star): How did they get there? Are they “pathological” cases or a typical outcome of planet formation?

The 20 or so cold Jupiters that have been discovered so far are thought to have either

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A good looker. When the Atacama Large Millimeter/submillimeter Array (ALMA) is completed in Chile later this year, it will consist of 54 12-m and 12 7-m antennae, spread over an area of 16 km in diameter.

migrated to their present location by gravitational interaction with other planets or the disk, or to have been formed in place through gravitational collapse (6). The planets in the two Dent *et al.* scenarios argue for migration, because Mars-size planets are too small to be formed by typical gravitational collapse models (9), and the alternative scenario requires the planet to migrate in order to produce the enhanced collision rate of icy bodies (10).

The planet signature found by Dent *et al.* is much more subtle than the massive warp caused by β Pic b. What made the mapping of CO in the disk possible is the giant submillimeter radio observatory being constructed in the Atacama desert in northern Chile, named the Atacama Large Millimeter/submillimeter Array (ALMA). Even in its half-completed state a year ago, ALMA was already the most powerful observatory of its kind. In the years to come, with a fully operational ALMA, we will no doubt witness an explosion of detailed observations of disk structure, giving another measure of how diverse planetary systems can be.

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MATERIALS SCIENCE

Why Mineral Interfaces Matter

Andrew Putnis

Throughout Earth, rocks respond to changing physical and chemical conditions by converting one rock type to another. These conversions have conventionally been described in terms of solid-state mechanisms, in which new minerals nucleate and grow through exchange of elements by diffusion. The slow rates of solid-state diffusion suggested geological time scales for these processes. However, rocks in Earth's crust are not dry (1), and even very low concentrations of aqueous solutions can increase reaction rates substantially (2). In the presence of a fluid phase, mineral conversions turn out to proceed not via solid-

state diffusion but through dissolution and recrystallization at the mineral-fluid interface (3). Well beyond mineralogy, these insights may prove useful in developing new methods of materials synthesis, for carbon removal from the atmosphere, and for safe nuclear waste storage.

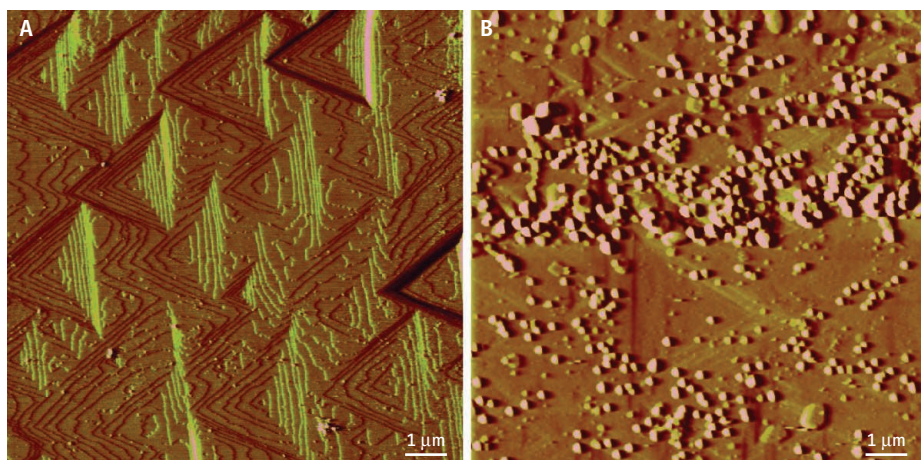
Depending on the chemical composition of the aqueous solution, the dissolution of even a few monolayers of a mineral surface can supersaturate an interfacial layer of solution with respect to another solid phase. If this phase can nucleate and grow on the parent substrate, inside the diffusion distance in the fluid, a feedback between the dissolution rate and the growth rate couples the two processes. This feedback allows the parent solid phase to be replaced by a product phase with

Reactions at mineral-fluid interfaces play a key role in processes ranging from the deep Earth to materials synthesis and nuclear waste storage.

different composition while maintaining its original dimensions.

Such a “pseudomorphic” replacement was long thought to require a solid-state mechanism, especially given that crystallographic orientations from the parent can be transferred to the product phase. However, an interface-coupled dissolution-precipitation mechanism (4) can transfer crystallographic information via structural matching (epitaxy) when the product nucleates on the parent substrate. Depending on the degree of structural matching, a single parent crystal can be pseudomorphically replaced by a single crystal or a polycrystalline product through an interface-coupled mechanism.

For the complete replacement of one phase by another, surface coverage by the



Interfacial processes. (A) Carbonated solution produces triangular dissolution etch-pits on the surface of brucite, $\text{Mg}(\text{OH})_2$, reflecting the trigonal symmetry of this mineral. Each step in this atomic force microscope image is a molecular monolayer (3). (B) Brucite dissolution is coupled to the precipitation of nanoparticles of a magnesium carbonate phase (13).

new phase should not armor the parent from further reaction; the product must have porosity and hence permeability to allow continued fluid access to the parent phase. The generation of porosity depends on the relative molar volumes of parent and product phases, as well as their relative solubility in the interfacial fluid. The product phase is more stable than the parent (and hence less soluble in the specific aqueous solution), meaning that more of the parent is dissolved than product precipitated within the same external dimensions; this generates porosity. Because of the close coupling between dissolution and precipitation, only the interfacial layer of fluid needs to be supersaturated with respect to the product phase.

The interaction of aqueous solutions with solids is ubiquitous throughout Earth, from chemical weathering at the surface to reactions deep in the crust (5). The dynamics of these large-scale processes ultimately depend on nanoscale mechanisms at the mineral-fluid interface, and the coupling of dissolution and precipitation forms the basis of our understanding of element transport in the crust. Understanding the nanoscale mechanisms of fluid-mineral interaction can be exploited to discover geo-inspired routes for the synthesis of functional materials.

For example, Reboul *et al.* have exploited the inheritance of morphology during a replacement process, which can be controlled in the laboratory by parameters such as fluid composition and temperature, to synthesize predefined architectures of porous coordination polymer (PCP) crystal networks (6). The method relies on pseudomorphically replacing a porous, three-dimensionally patterned aluminum (Al) oxide parent, which acts as

both the metal source and the architecture-directing agent, by an identically shaped Al-PCP product. The local dissolution of the Al oxide by reaction with a solution containing organic ligands provides the metal ions that supersaturate the interfacial solution with respect to the Al-based PCP. An advantage of this method is the hierarchical nature of the porosity in the final product, combining the porosity of the original alumina architecture with the secondary porosity in the PCP due to the replacement mechanism. The method holds considerable promise for the design of porous coordination polymer architectures for use in catalysis, sensing, and adsorption separation (6).

Xia *et al.* (7) have used a similar strategy to synthesize three-dimensional ordered arrays of nanocrystals of the zeolite analcime ($\text{NaAlSi}_2\text{O}_6 \cdot \text{H}_2\text{O}$) by pseudomorphic replacement of natural leucite (KAlSi_2O_6), which contains an inherent three-dimensional ordered pattern of nanosized lamellar twins. After reaction in NaCl solutions, these patterns are preserved in the resulting array of analcime nanocrystals, the size of which can be tuned by changing the pH of the solution. The same authors have also used this method to synthesize complex metal sulfides with low thermal stability (8). At low temperatures, traditional synthesis from the elements is far too slow compared to pseudomorphically replacing a preexisting precursor sulfide using an appropriate aqueous solution.

The same principles may apply to carbon capture and storage by mineral carbonation. The idea is that reaction of carbonated fluids with magnesium- and calcium-rich rocks supplies metal ions by dissolution, resulting in the precipitation of a metal carbonate. For

example, dissolution of brucite, $\text{Mg}(\text{OH})_2$, a natural constituent of altered mantle rocks, is coupled to the precipitation of a magnesium carbonate phase (see the figure) (9). Understanding the chemical controls on this coupling is a necessary prerequisite to using this strategy of carbon capture and storage. During the dissolution of many silicate minerals by carbonated fluids, metal ions are preferentially released into solution. This results in the formation of silica-rich surface layers (“leached layers”) that may have a substantial effect on the reaction kinetics (10).

The differential extraction of metal ions from minerals by treatment with aqueous solutions (leaching) is routinely used in industrial processes (e.g., the production of titanium dioxide from ilmenite, FeTiO_3). The mechanism of leaching has been the subject of debate, especially in relation to chemical weathering on Earth, with the consensus moving toward a dissolution-precipitation mechanism as opposed to diffusional exchange (11).

The same questions concern the mechanism of aqueous alteration of glass used to encapsulate radioactive nuclear waste. The currently accepted models, based on diffusional exchange of ions between the glass and the solution, are being challenged by new models based on coupled dissolution-precipitation mechanisms (12).

Understanding fluid-solid interaction mechanisms is important to diverse fields of Earth science, materials science, and chemistry. Knowledge of the chemical controls on the feedback mechanisms that couple dissolution and precipitation are a necessary prerequisite for predictive numerical modeling. Currently, this knowledge is limited. Studying mineral-fluid reactions in synthetic systems, as well as in Earth’s natural laboratory, will continue to supply some of the answers.

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BIOCHEMISTRY

The Resolution Revolution

Werner Kühlbrandt

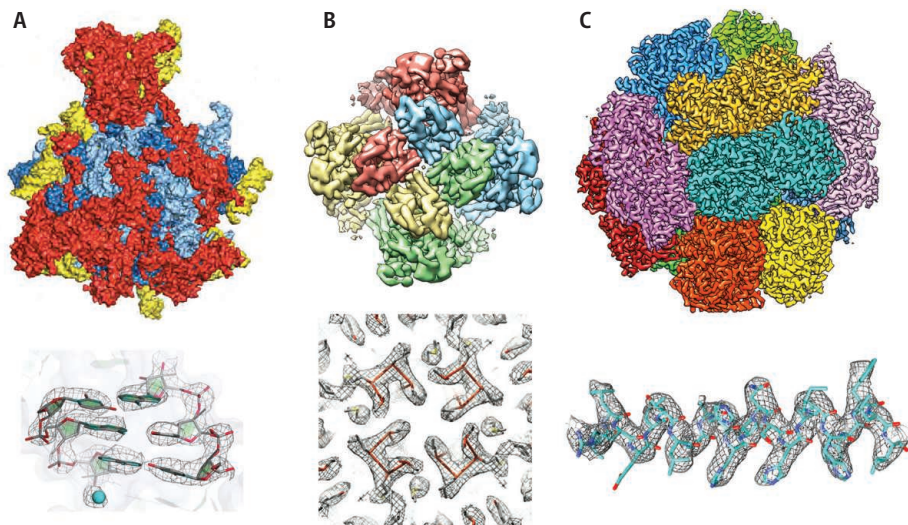
Precise knowledge of the structure of macromolecules in the cell is essential for understanding how they function. Structures of large macromolecules can now be obtained at near-atomic resolution by averaging thousands of electron microscope images recorded before radiation damage accumulates. This is what Amunts *et al.* have done in their research article on page 1485 of this issue (1), reporting the structure of the large subunit of the mitochondrial ribosome at 3.2 Å resolution by electron cryo-microscopy (cryo-EM). Together with other recent high-resolution cryo-EM structures (2–4) (see the figure), this achievement heralds the beginning of a new era in molecular biology, where structures at near-atomic resolution are no longer the prerogative of x-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy.

Ribosomes are ancient, massive protein-RNA complexes that translate the linear genetic code into three-dimensional proteins. Mitochondria—semi-autonomous organelles that supply the cell with energy—have their own ribosomes, which closely resemble those of their bacterial ancestors. Many antibiotics, such as erythromycin, inhibit growth of bacteria by blocking the translation machinery of bacterial ribosomes. When designing new antibiotics, it is essential that they do not also block the mitochondrial ribosomes. For this it is of great value to know the detailed structures of both. The structures of other ribosomes have been determined by x-ray crystallography (5, 6). In determining the high-resolution structure of the mitochondrial ribosome by cryo-EM, Amunts *et al.* achieve something that, less than a year ago, few would have thought possible.

To be able to do this without crystals is nothing short of a revolution, made possible by a new generation of electron detectors of unprecedented speed and sensitivity. The new sensors detect electrons directly, rather than first converting them into photons that are then reconverted into photoelectrons. The latter is what the widely used CCD (charge-coupled device) cameras do, but they do not perform well at high resolution.

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Advances in detector technology and image processing are yielding high-resolution electron cryo-microscopy structures of biomolecules.



Near-atomic resolution with cryo-EM. (A) The large subunit of the yeast mitochondrial ribosome at 3.2 Å reported by Amunts *et al.* In the detailed view below, the base pairs of an RNA double helix and a magnesium ion (blue) are clearly resolved. (B) TRPV1 ion channel at 3.4 Å (2), with a detailed view of residues lining the ion pore on the four-fold axis of the tetrameric channel. (C) F_{420} -reducing [NiFe] hydrogenase at 3.36 Å (3). The detail shows an α helix in the FrhA subunit with resolved side chains. The maps are not drawn to scale.

Photographic film works in principle much better for high-resolution imaging, but is incompatible with rapid electronic readout and high data throughput, which are increasingly essential.

Some 10 years ago, Henderson and Faruqi realized that it should be possible to design a sensor that detects electrons directly and that combines the advantages of CCD cameras and film (7). They and two competing teams (8) have since developed detectors that use essentially the same active pixel sensor technology as the camera chips in most cell phones. However, cell phone chips cannot be used in the electron microscope because the intense electron beam would destroy them instantly. The sensors therefore had to be made radiation-hard. Second, the pixels needed to be much larger to prevent the energy-rich electrons from exciting more than one pixel at a time. Third, the camera chip, complete with readout electronics in each of its 1.6 million pixels, had to be very thin, otherwise electron scattering would blur the image and compromise resolution. Current sensors are about half as thick as a sheet of paper.

Cryo-EM requires only small amounts of material. Samples that cannot be isolated in large enough quantities for x-ray crystallography can now yield high-resolution struc-

tures. The same holds for heterogeneous samples or flexible complexes that do not crystallize readily, because cryo-EM images of different particles or conformations are easily separated at the image processing stage.

The new detectors offer another decisive advantage: Their fast readout makes it possible to compensate small movements that inevitably happen when the electron beam strikes the thin, unsupported cryo-sample. Before the new cameras were developed, blurring by beam-induced movement was an insidious, seemingly insurmountable problem. Now, dozens of images of one area are taken in rapid succession, and beam-induced movements are detected and reversed in the computer. The impact of this deblurring is similarly dramatic as that of the Hubble telescope in astronomy, although the blurring is caused by different effects in the two cases.

The new cameras also promise a major breakthrough in electron cryo-tomography, which images three-dimensional volumes of whole cells, cell slices, or cellular compartments, such as mitochondria (9). Averaging of recognizable molecular features in tomographic volumes is already revealing subnanometer detail even with standard CCD cameras (10). The new detectors are bound to make an enormous difference in this area.

Concurrently with the new cameras, powerful maximum likelihood image processing routines became available. These routines define reliable and objective criteria for averaging tens or hundreds of thousands of single-particle images, as is necessary to achieve high resolution (11). This combination of advanced detectors and software now produces cryo-EM structures that look, in terms of clarity and map definition, considerably better than x-ray structures at the same nominal resolution, owing to the high quality of the phase information contained in cryo-EM images.

Does the resolution revolution in cryo-EM mean that the era of x-ray protein crystallography (12) is coming to an end? Definitely not. For the foreseeable future, small proteins—in

cryo-EM, anything below 100 kD counts as small—and resolutions of 2 Å or better will remain the domain of x-rays. But for large, fragile, or flexible structures (such as membrane protein complexes) that are difficult to prepare yet hold the key to central biomedical questions, the new technology is a major breakthrough. In the future, it may no longer be necessary to crystallize large, well-defined complexes such as ribosomes. Instead, their structures can be determined elegantly and quickly by cryo-EM. These are exciting times.

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APPLIED PHYSICS

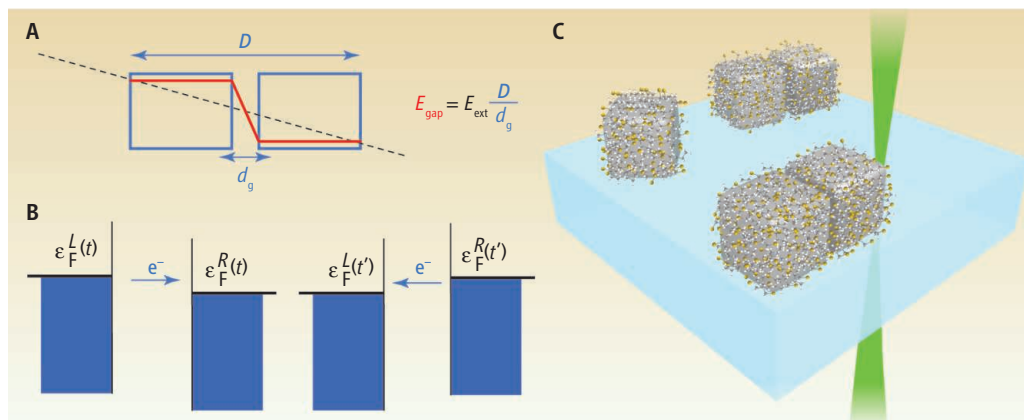
Molecular Tuning of Quantum Plasmon Resonances

Peter Nordlander

Metallic nanoparticles exhibit plasmon resonances, which are collective, coherent oscillations of their conduction electrons that can couple very efficiently to light. Originally a subfield of condensed-matter physics, the past decade has seen tremendous growth of plasmonics as an interdisciplinary field spanning chemistry, materials science, and biology. On page 1496 of this issue, Tan *et al.* (1) discuss an experiment that will almost certainly further fuel this growth—the coupling of plasmon excitations to molecular conduction. The merging of plasmonics with molecular electronics promises both novel fundamental discoveries and new applications.

The energy of the plasmon resonances depends strongly on nanostructure shape and composition. Given the emergence of highly precise nanofabrication methods, it is possible to design nanostructures that have plasmon resonances ranging from the ultraviolet into the infrared. Among the many important applications of the light-harvesting proper-

The tuning of nanostructure plasmon resonances with bridging molecules offers opportunities for both plasmonics and molecular electronics.



Plasmon-induced electron tunneling. (A) The electric potential associated with the incident radiation field (black dashed line) is screened by the nanoparticles and creates an enhanced local field in the gap (solid red line) determined by the structural parameters (blue) D and d_g . (B) The field in the junction shifts the relative positions of the Fermi levels ϵ_F of the left and right particles as a function of time t and enables electrons to transfer between nanoparticles in each cycle of the incident radiation. (C) A schematic of the molecular junction used in the present experiment shows the planar junction geometry and interrogation by a light beam (green) that allows a large number of molecules to serve as conduction channels for electrons.

ties of plasmonic nanoparticles, perhaps the most mature is the use of the large plasmon-induced enhancements in local electromagnetic fields near the surfaces of the nanoparticles for surface-enhanced spectroscopies (2). Amplified local fields can enhance the probabilities for molecular transitions by many orders of magnitude. In nonlinear spectroscopies such as Raman scattering, the signal scales as the fourth power of the local electric field across the molecule.

Nanostructures with sharp protrusions naturally induce large local field enhancements, but the optimal structures for local field enhancements consist of a pair of metallic nanoparticles separated by a nanometer-scale gap (a “plasmonic dimer”). Several studies have demonstrated that the local field enhancements in the “hot spots” in the gaps of dimers can be sufficiently strong that the Raman scattering from an individual molecule can be detected (3). Classical electro-

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magnetic theory predicts that the local field enhancement in the junction of a plasmonic dimer increases monotonically with decreasing gap width. This property can be understood in a very simplified fashion as a “lightning rod” effect (see the figure, panel A). In the long wavelength regime, the nanoparticles are essentially equipotential; the potential drop from the electric component of the incident field across the dimer occurs only in the gap (4). For a dimer of length D with a gap of width d_g , the local field enhancement will scale as D/d_g and can be made very large by fabricating structures with large D and small d_g .

The large fields in the gap also result in a voltage drop between the nanoparticles that shifts the relative position of the Fermi energies ϵ_F of the nanoparticles (see the figure, panel B). In principle, these shifts allow electrons to transfer back and forth between the nanoparticles. This plasmon mode, referred to as the charge-transfer plasmon (CTP), coexists with the standard nonconductively coupled dimer plasmons but is distinct and appears at different frequencies because the local fields in the junctions are altered by the charge transport across the junction (5). For a vacuum junction, the tunneling matrix elements, which determine the transition rates of electrons between the two nanoparticles, decrease exponentially with increasing d_g , and the CTP can only be observed for gaps a few angstroms in width. The creation of dimers with such narrow gaps is experimentally challenging but has recently been accomplished and has enabled the experimental observation of well-defined CTPs (6, 7).

Theoretical calculations have predicted that if the nanoscale gap is filled by a conductive medium such as molecules, the tunneling transition matrix element will no longer exhibit an exponential dependence on d_g and the CTP may be observed at larger d_g (8, 9). The experimental verification of these predictions is a challenging task, but Tan *et al.* were able to nanofabricate dimers with extremely small gap widths, as well as assemble molecules that span the gap. By fabricating a silver nanocube dimer with very flat surfaces (see the figure, panel C), the junction area became large and uniform and could accommodate a sufficiently large number of conducting molecules. The CTP was observed for junction widths greater than 1 nm.

These results not only allow quantum plasmonics to be studied with structures that have substantially larger gap widths than in previous experiments but also bridge two vibrant subfields of nanoscience, plasmonics, and

molecular electronics. Tan *et al.* show that the CTP depends on the type of molecule used, thus firmly establishing that its electronic properties are a key parameter in determining the optical frequency transport response of the nanoscale interparticle junction.

Molecular tunnel junctions are normally characterizing with direct current (dc) or low-frequency alternating current conductivity measurements. In the present experiment, the molecular conductance was probed instead at optical frequencies determined by the collective resonance of the plasmonic dimer. At high frequencies, additional intramolecular processes not present in dc transport, such as electron-electron and electron-vibrational scattering, could strongly influence molecular conductance. By exploiting the facile geometric tunability of plasmonic antennas, it should be possible to measure molecular

conductances at much higher frequencies. This experiment by Tan *et al.* also paves the way for novel device and molecular sensing applications. The conductance of a molecule can be switched or modulated, for example, by the application of an external dc field or by chemical reaction with another molecule.

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CANCER

Cholesterol and Cancer, in the Balance

Sandrine Silvente-Poirot and Marc Poirot

Cholesterol metabolites can promote or suppress breast cancer, raising questions about how therapies might disrupt this balance.

Mammalian cells synthesize cholesterol through a series of 21 enzymatic steps, generating numerous metabolites that are involved in the control of physiological and developmental processes. Cholesterol itself is the precursor of steroid hormones and sterols, the latter of which can be further modified into molecules that induce specific biological responses. Epidemiological studies have investigated the role of cholesterol in breast cancer risk, with contradictory findings. Recent studies, however, linking cholesterol metabolism to breast cancer may provide some insights. Certain cholesterol metabolites can promote (1, 2) or suppress (3) breast cancer. This raises the important question of how to regulate or inhibit the cholesterol metabolic pathway, and at which steps, in a therapeutic approach to cancer.

Cholesterol is a unique lipid, essential for membrane biogenesis, cell proliferation, and cell differentiation (4). It is provided by the diet but is also mainly synthesized by the liver in humans and distributed throughout the

body via low-density lipoprotein (LDL) and high-density lipoprotein (HDL) transporters.

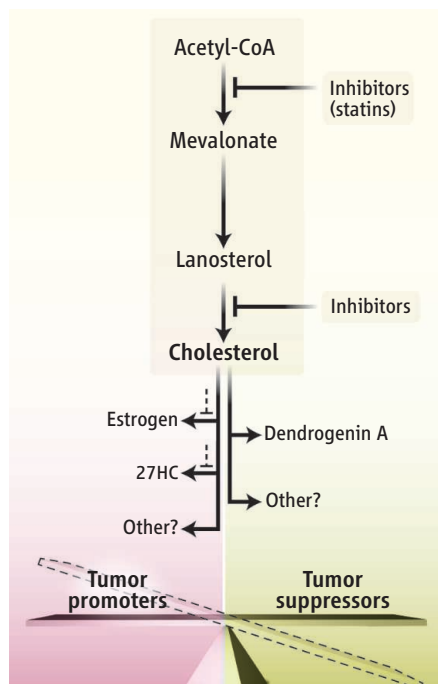
Cancer has been associated with cholesterol, as it is the obligatory precursor of steroid hormones that are involved in tumor promotion (estrogens, androgens) as well as tumor death (glucocorticoids). Oncogenic processes enable cancer cells to synthesize their own cholesterol, which can be further metabolized to support their rapid proliferation. But contradictory results of epidemiologic studies make conclusions difficult regarding breast cancer. Thus, it is still not clear whether total cholesterol, HDL, or LDL can predict the occurrence of breast cancer. Many studies have investigated whether breast cancer risk is affected by blocking cholesterol synthesis with anticholesterol drugs such as statins, which inhibit the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase or HMGCR). Here too, the findings are inconsistent. Statin use is associated with both an increased and decreased risk of breast cancer, and other studies report no association at all. A recent study has found that long-term (10 years) treatment with statins doubled the risk of invasive ductal

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carcinoma and invasive lobular carcinoma among postmenopausal women (5)—a disconcerting finding given that statins are commonly taken for chronic, long-term use.

Investigations at the laboratory level have revealed a more complex map of the influence of cholesterol metabolism on the promotion or suppression of breast cancer that could account for these conflicting studies. Hypercholesterolemia was shown to promote mammary tumor growth and invasiveness in several mouse transgenic models (6–8), which suggested that cholesterol or its metabolites promote breast cancer. Indeed, recent studies show that the cholesterol metabolite 27-hydroxycholesterol (27HC) spurs tumor growth by interacting with the estrogen receptor in several animal models of breast cancer (1, 2). 27HC also promotes breast cancer metastasis by interacting with liver X receptor, a transcription factor (1). These observations seem to have clinical relevance because increased amounts of the enzyme that eliminates 27HC [called oxysterol and steroid 7- α -hydroxylase (CYP7B1)] in human breast cancer samples were associated with improved patient survival (1, 2), and increased amounts of the enzyme that converts cholesterol to 27HC [called sterol 27-hydroxylase (CYP27A1)] were observed in more aggressive mammary tumors. Tumor samples (that express the estrogen receptor) from breast cancer patients showed a higher content of 27HC compared to normal breast tissue in the patients and relative to the same tissues in cancer-free controls. This shows that there is a deregulation of 27HC production during carcinogenesis toward a higher production of the metabolite; this increase is independent of the amount of circulating cholesterol (2). Blocking 27HC production, using an inhibitor of CYP27A1 or a statin, attenuated hypercholesterolemia-promoted tumor growth in a mouse model (1). These studies highlight that a cholesterol metabolite is a tumor promoter in breast cancer (in the presence of the estrogen receptor).

The deregulation of metabolite production at different points in the cholesterol biosynthesis pathway has been reported to promote breast cancer or cause resistance to therapies in different *in vitro* and *in vivo* models. Thus, mucin1, a glycoprotein that is aberrantly overexpressed in numerous cancers, induces a lipid and sterol metabolism transcriptional signature in breast cancer tissue that is predictive of resistance to tamoxifen treatment, the gold standard for therapy and prevention of estrogen receptor-expressing breast cancer (9). Similarly, mutant forms of the tumor suppressor protein (and transcrip-



Cholesterol and breast cancer. Deregulations along the cholesterol metabolic pathway may favor the accumulation of metabolites with tumor-promoting activity (such as 27HC) but may also be detrimental to the formation of other metabolites that are beneficial to cell integrity and differentiation (such as dendrogenin A).

tion factor) P53, which are present in 50% of all cancers, increase the expression of several genes involved in the synthesis of sterols that are associated with the growth and invasiveness of breast cancer; this correlates with a low probability of breast cancer patient survival (10). Among these genes are those encoding 7-dehydrocholesterol reductase (catalyzes the production of cholesterol from 7-dehydrocholesterol), a subunit of cholesterol epoxide hydrolase (catalyzes the hydration of cholesterol-5,6-epoxides), and acyl-coenzyme A cholesterol acyltransferase 2 (catalyzes the esterification of cholesterol) (9, 10). These three enzymes have been characterized as therapeutic off-targets of tamoxifen in addition to the estrogen receptor (11).

Inhibition of cholesterol biosynthesis at different post-lanosterol steps also has an impact on tumor growth, cell proliferation, and the induction of tumor cell differentiation (12). Depletion of enzymes that lead to the accumulation of sterols (specifically, the lack of sterol-C4-methyl oxidase and NADP-dependent steroid dehydrogenase-like enzymes results in the accumulation of 4-methylsterols) markedly sensitizes cancer cell lines and tumor xenografts to epidermal growth factor receptor-targeting drugs that are used to treat a number of cancers,

including breast cancer (13, 14). Inhibition of cholesterol epoxide hydrolase results in the accumulation of its cholesterol oxide substrates (cholesterol-5,6-epoxides), and this contributes to the antitumor and cell differentiation actions of tamoxifen in breast cancer cells (15).

Yet another recent study reveals a cholesterol metabolite that suppresses breast cancer. Dendrogenin A is an amino-oxysterol metabolite that arises from cholesterol-5,6-epoxides and histamine in mammals (3). It triggers breast cancer cell redifferentiation (recovery of normal physiological functions) both *in vitro* and *in vivo* and improves survival in animal models. Interestingly, the amount of this cholesterol metabolite decreased in tumors from breast cancer patients relative to normal matched tissues, suggesting a deregulation of dendrogenin A biosynthesis during carcinogenesis. Its properties and decreased presence in tumors suggest a physiological function in maintaining cell integrity and differentiation. It is not known whether the amounts of circulating cholesterol affect the biosynthesis of dendrogenin A, and the enzymes involved in its biosynthesis and catabolism are yet to be identified.

Perhaps a balance in the production of tumor promoters such as 27HC and tumor suppressors such as dendrogenin A regulates breast tumor development (see the figure). Because other sterol derivatives may have such opposing properties, it is important to quantitatively profile sterols and oxysterols in the blood of patients, in tumor cells, and in cells of the tumor microenvironment, and also to measure the expression of genes and enzymes controlling the “sterolome.” This may lead to better targeting of these branches of the cholesterol metabolic pathways and to the development of new therapeutic approaches for breast cancer and possibly other cancers.

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ANNUAL MEETING

AAAS Speakers Seek Ways to Expand Science Communication

CHICAGO — At the AAAS Annual Meeting, actor and Stony Brook University professor Alan Alda delivered a slew of punchlines about ways for scientists to get “beyond a blind date” with the public. But his concluding remarks hit a more serious note. “If you want commitment, you’re going to have to listen to each other,” he said. “If we want commitment to take place, we’re going to have to get aware of what’s happening in the other person’s mind.”

The critical need for science communication was a theme found throughout the 2014 meeting, which drew more than 7000 scientists, educators, journalists, and students from nearly 60 countries. In symposia and workshops, participants discussed ways to expand engagement with the public, including reaching out to groups that sometimes stand apart from science’s mainstream.

Evangelical Christians in the United States are one such group, but new survey results released at the meeting suggest that the relationship between evangelicals and the scientific community may be less combative than is commonly portrayed. Among more than 10,000 people in the study, nearly half of the evangelicals surveyed said they felt science and religion were in a collaborative rather than confrontational relationship. And nearly a third of survey participants said they were very interested in new scientific discoveries.

AAAS’s Dialogue on Science, Ethics and Religion (DoSER) commissioned the survey as part of a multiyear project that will include several regional workshops and a 2015 national workshop that will bring together leaders from local science and evangelical communities, according to DoSER Director Jennifer Wiseman. “There are a lot of commonalities between much of what religious communities are interested in about science and what scientists are also interested in,” Wiseman said, “and that often involves science and technology for the improvement of life.”

Minority communities may feel underserved by current science and technology engagement efforts, said Danielle N. Lee, a postdoctoral researcher at Cornell University and a blogger for *Scientific American*.

At the meeting’s seminar on communicating science, Lee noted that publications in the ethnic minority press rarely have science sections or dedicated science reporters. She suggested that social media might be a better alternative for reaching African Americans and Latinos. “Because [science] is not universal, you have to find the language to communicate with different audiences.”

This type of outreach affects the views that people in minority communities have of scientists, Lee said, and those views translate into classrooms and the science, technology, engineering, and mathematics workforce.

Whether told via social media or at more formal events, scientists’ personal stories also can be an important source of encouragement for students and early-career researchers from underrepresented groups. At the meeting’s standing-room-only Women and Minority Scientists Networking Breakfast, organized by AAAS Education and Human Resources, several participants gave examples of how hearing other minority scientists



describe their own struggles had encouraged them to persevere. “Keep telling your stories, because you never know who you may be inspiring,” one graduate student urged the others in the room.

Other meeting speakers, including former AAAS President Phillip A. Sharp, emphasized the need for better communication between scientists to improve research itself. In his address to open the Chicago meeting, Sharp, who is now chair of the AAAS Board, said that biological and physical scientists and engineers must learn how to communicate and work collaboratively to produce the sort of interdisciplinary projects that will be “the blueprint for future innovations.”

— Sarah Zielinski and Becky Ham



Finding their audience. Danielle Lee (above) and Alan Alda said that scientists must be sensitive to the diverse backgrounds and viewpoints of the people they seek to communicate with.

AAAS Launches Strategic Transformation for the 21st Century

AAAS has embarked on a far-reaching effort to make the robust organization even stronger, by enhancing its engagement with its members and enabling the *Science* family of journals to provide leadership in science communication in the dynamic, multimedia landscape of the future.

“From every perspective, AAAS is in great shape, but every organization needs to periodically evaluate its role going forward. For the last 2 years, the AAAS Board of Directors has been engaged in a long-term strategic planning process, the goal of which is to position AAAS to remain strong for many years to come,” said Alan I. Leshner, chief executive officer of AAAS and executive publisher of *Science*.

The initiative arose from an understanding that the AAAS of the future must reflect the seismic shifts under way in how science functions and interfaces with society.

“Communication among scientists is changing rapidly, and *Science* and other communication venues of tomorrow will be quite different. Similarly, members will engage with AAAS in new ways. It is

wonderful that AAAS is moving into this new world from a position of excellence and financial strength,” said Phillip Sharp, chair of the AAAS Board of Directors and Institute Professor at the Koch Institute of Integrative Cancer Research at the Massachusetts Institute of Technology.

AAAS has surveyed current and prospective members, as well as customers of its many programs, to learn how the association can best serve them. While the core mission and goals of AAAS will stay the same, the surveys pointed in two new directions: moving away from print-centric publishing to serving as a multimedia communication organization, and better engaging with and providing a wider array of useful services to members and to the broader society interested in science.

AAAS has already initiated internal reorganizations to expand its digital capabilities under the direction of Robert Covey, in the newly created position of digital media officer. Covey will work in concert with *Science*'s editor-in-chief Marcia McNutt and others to develop a vibrant, multiplatform

experience for the *Science* family of journals, including a refreshment of their design. In addition, AAAS will launch a new, online platform through which anyone in the scientific community can gather to engage on any topic of interest, regardless of discipline, affiliation, or geography.

Science policy and advocacy is another area in which the organization will strengthen and expand engagement with its membership. “The feedback we’ve gotten from members is that they want AAAS to take a more visible role in speaking up for science, both on the Hill and in the public commons,” said Joanne Carney, director of the Office of Government Relations. In a survey that produced 3300 responses, over 80% of respondents felt that it was very important for AAAS to advocate more for science, technology, mathematics, and engineering in the policy arena.

AAAS has long had a strong science advocacy presence, but will expand it significantly. Its latest efforts have included protecting the integrity of science from distortion and misuse, championing adequate federal funding for research and development, presenting the science that underpins a range of policy issues such as climate change, and addressing the importance of teaching evolution. AAAS will develop approaches that allow members to take part in these efforts, focusing on federal policy with secondary efforts at the state level.

Career advice and support also came in as a top priority for members. Under the new initiative, AAAS/*Science* will work to become a “one-stop shop” for all nondisciplinary career development.

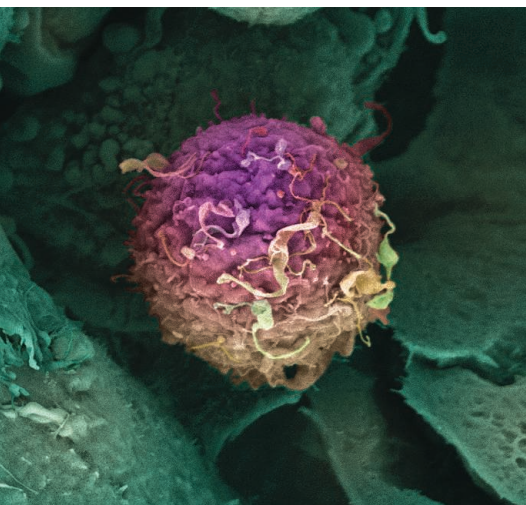
At least 50 different career-related projects are currently active across the association, including offerings from *Science*, *Science* Careers, MemberCentral, Education and Human Resources, the Center for Public Engagement With Science and Technology, and other programs, but they will be enhanced and integrated, with a focus on member needs and scaling for wider use, said Bill Moran, director of global collaboration and custom publishing and leader of a taskforce on these new program approaches. While many of the current services are particularly useful to postdoctoral researchers in search of permanent positions, AAAS also will become a resource for members at all stages of their careers, providing support “from before their first job all the way to their last job,” Moran said.

Input is welcome and can be sent to aleshner@aaas.org.



Kids Get Their Hands on Science at Family Science Days

CHICAGO — Over 3300 children and adults from across Chicagoland came to Family Science Days at the AAAS Annual Meeting, where they explored robots, giant bubbles, endangered animal pelts, and much more. Free and open to the public, Family Science Days featured hands-on demonstrations, shows, and other activities appropriate for K-12 children and their families. “A little girl told us that she ‘played so much science,’ which is a nice way to describe our desired effect,” said Jeanne Braha, public engagement manager at AAAS.



INTRODUCTION

A Race Still Unfinished

FEW SCIENTIFIC ENDEAVORS CAPTURED AS MUCH PUBLIC INTEREST AS THE RACE TO identify *BRCA1*, a gene responsible for inherited predisposition to breast and ovarian cancers. The search culminated in a 1994 *Science* paper reporting the isolation of *BRCA1* by positional cloning.* Isolation of the related cancer predisposition gene *BRCA2* followed soon after.† Twenty years later, the *BRCA* genes continue to make headlines, sparking a Supreme Court decision on the legality of gene patenting and intense debates on the ethics of genetic testing.

In this special section of *Science*, expert contributors retrace the long and tortuous path leading to the mapping and identification of the *BRCA1* gene; discuss the ways in which *BRCA* mutation status has been integrated into the clinical management of patients in high-risk families; and highlight the role of the *BRCA* proteins in preserving the structural and numerical integrity of chromosomes throughout the cell cycle, a function that may explain their tumor suppressor activity.

Science's News team explores many of the issues that have arisen since the discovery of the *BRCA* genes. Advances in molecular medicine have brought new options for treatment and prevention, driving down mortality rates in wealthy countries. But in poor countries, shut out from these advances, mortality rates remain disproportionately high. Widespread breast screening has led to early detection and a new dilemma: whether to aggressively treat noncancerous lesions that may never become invasive or whether to “watch and wait.” Since 1994, dozens more genes have been found that increase a woman’s risk of hereditary breast cancer, but by how much is uncertain, confounding already complicated issues in genetic testing and counseling. Throughout this explosion of research, advocacy groups have been a powerful force in boosting funding and shaping the agenda, and they continue pushing for increasingly ambitious, some say unrealistic, goals.

Clearly much more needs to be done—both more research and better ways to get existing treatments to women who need them. The discovery of the *BRCA* genes was transformative. More than a million women and men have been tested for *BRCA1* and *BRCA2* mutations in the past 20 years, and there is no doubt that the test has saved lives. But mutations in these two genes account for only 5 to 10% of breast cancer cases in the general population, and worldwide, breast cancer remains the most common and most deadly cancer in women. Is it time to start a new race?

— PAULA KIBERSTIS AND LESLIE ROBERTS

*Y. Miki et al., *Science* 266, 66 (1994). †R. Wooster et al., *Nature* 378, 789 (1995).

Breast Cancer

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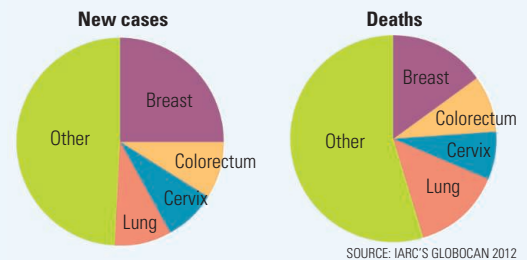
Science

Breast Cancer: A World of Differences

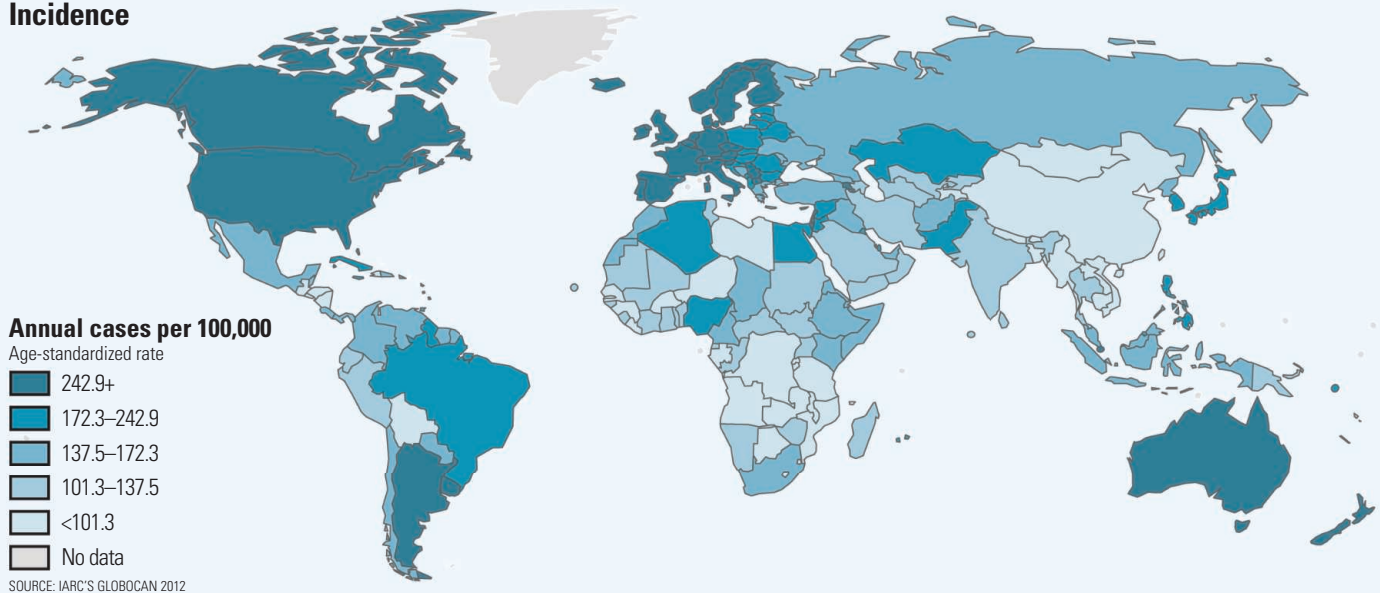
Worldwide, breast cancer is both the most common and the most deadly cancer in women. Incidence is highest in Europe and the Americas, although cases are on the rise in Africa and Asia. Scientific advances have given wealthy countries promising new means of prevention and treatment, driving mortality rates steadily downward over the last decade. But poor countries have not shared in the mortality decline. The factors influencing a woman's survival—from cultural and economic barriers to care to the genetic basis of the disease itself—vary dramatically across the world.

Reported and written by Kelly Servick

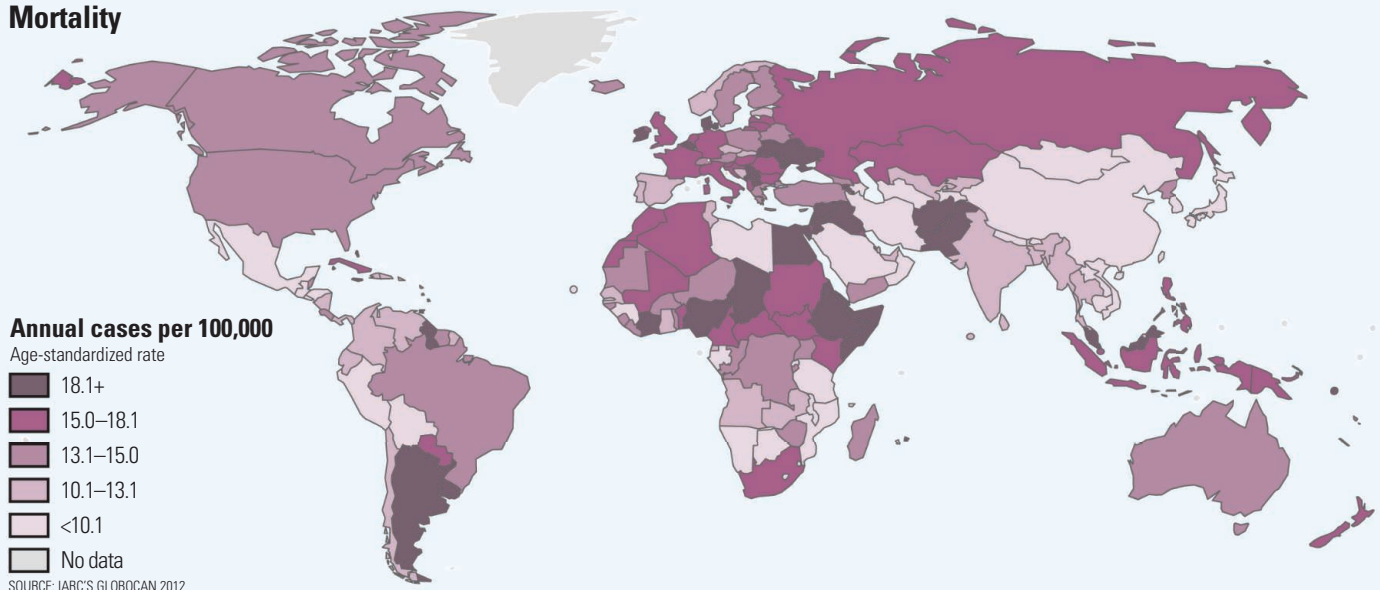
Cancer Incidence/Mortality in Women Worldwide

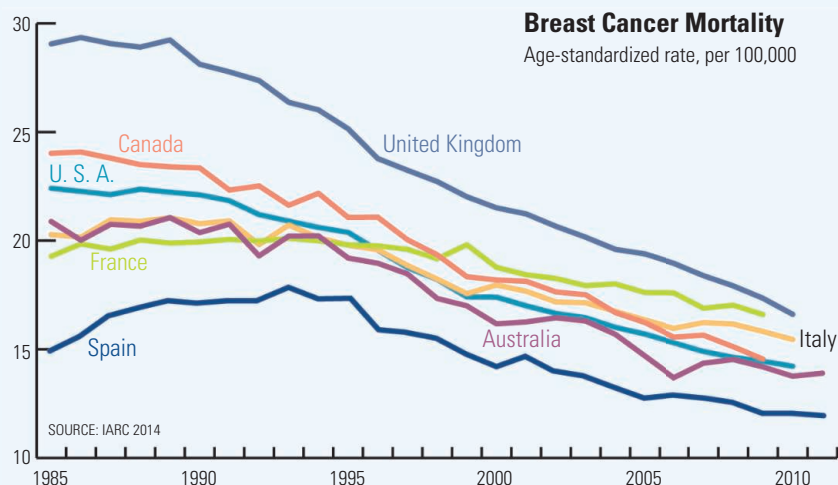


Incidence



Mortality





Big Gains in Wealthy Countries

Molecular medicine has reshaped the way the disease is treated. In the 1960s, researchers discovered that some breast cancer cells respond to growth signals from estrogen. Tamoxifen was shown to block estrogen receptors and inhibit tumor growth. Approved in 1977, it is now the gold standard for treating so-called ER-positive tumors—roughly 75% of all cases. In the 1990s, trastuzumab was shown to slow growth in tumors that overexpress the gene for human epidermal growth factor receptor 2 (HER2/neu). The drug has cut mortality rates in half in the roughly 20% of patients with this tumor subtype. Widespread screening has probably helped lower mortality, although how much is controversial.

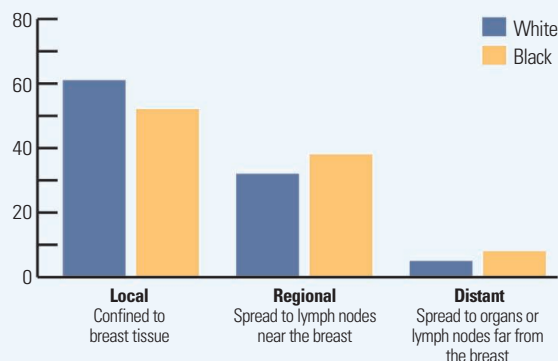
A Black-White Divide

Black women are less likely than white women in the United States to get breast cancer, yet more likely to die from it. Poverty and lack of insurance may play a role by preventing women from getting routine screenings or early treatment. But black women are also about three times more likely to develop the highly aggressive triple-negative

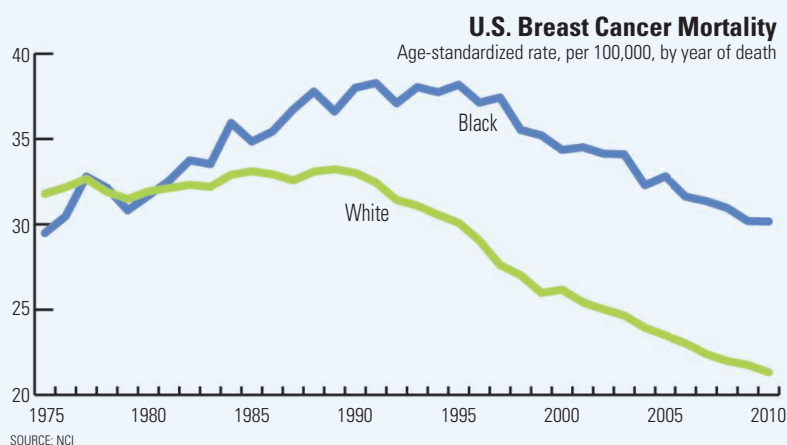
breast cancer, which occurs more often in younger women, who may not be getting routine mammograms. The triple-negative subtype has been linked to both lifestyle factors and genetic mutations. Some researchers suspect that the frequency of these mutations differs between women of Caucasian and African descent.

Stage Distribution at Diagnosis

By percentage, 2003–2009

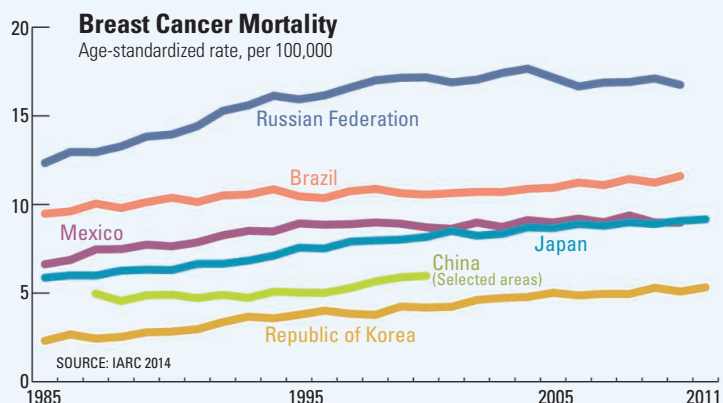


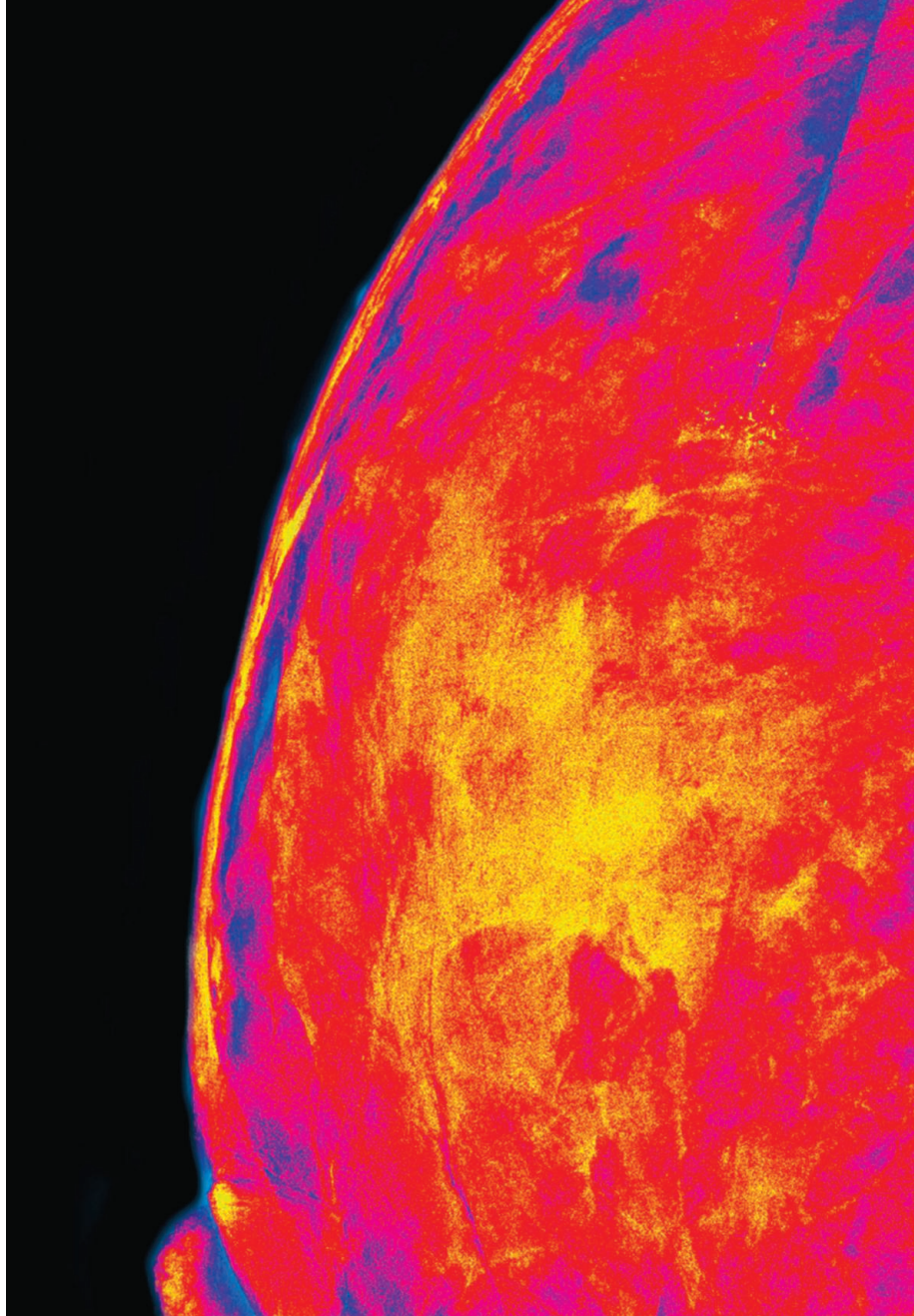
SOURCE: NATIONAL CANCER INSTITUTE



Worldwide, a Growing Toll

The incidence of breast cancer—like most cancers—is on the rise in middle-income countries such as Brazil and China, as populations increasingly adopt Western lifestyles. Higher rates of obesity and delayed childbearing raise the risk, as does longer life expectancy. And some high-income countries, particularly Russia, have gone against the trend of declining mortality in recent decades. In poor countries, mortality is disproportionately high because many women do not seek treatment for breast cancer or do so only when it is advanced. Clinics are often not equipped to treat it, and a one-size-fits-all regimen of surgery and chemotherapy remains the norm in many countries. Efforts to reduce the toll focus on the essentials: clinical screening and diagnostic guidelines, and supplying basic drugs to new clinics.





Stage zero. Each year, U.S. clinics detect more than 60,000 precancerous breast lesions known as DCIS; this scan shows a risky “high-grade” lesion (yellow).

olina, says that, with no decisive evidence, oncologists felt they had to treat each DCIS case as if it were invasive cancer. “We were removing all these breasts” to take out DCIS lesions, “the majority of which might never become clinically significant,” she says. Today, the diagnosis of DCIS usually leads to less radical surgery—removal of a few cubic centimeters of tissue (a “lumpectomy”), followed by radiation and hormone therapy. The cohort of U.S. women living with the diagnosis has risen steadily; one forecast estimates they will number 1 million by 2020.

But Hwang and other oncologists worry that women are still overtreated for DCIS that would never become life-threatening. They hope it will become possible to do more sophisticated analysis of each DCIS patient’s risk for invasive cancer and adjust treatment accordingly, avoiding radiation treatment, for example, or even in some cases surgery. Some, like oncologist Laura Esserman of the University of California, San Francisco (UCSF), have argued for years that the diagnosis should have a gentler name, one that omits “carcinoma.” She proposes calling these and similar slow-growing tissue irregularities “indolent lesions of epithelial origin,” or IDLE. The goal, Esserman says, is to let doctors and patients “take a step back” and be less aggressive with therapy.

Five years ago, leading physicians and researchers met at the Bethesda, Maryland, campus of the National Institutes of Health to review what’s known about DCIS. They concluded in a consensus document that it would be worth considering a less “anxiety-producing” name, as well as “less therapeutic intervention” if it could be done without increasing the risk of subsequent cancer. Testing new approaches to treatment is difficult, given that current practice is judged a success: Ninety-eight percent of U.S. women treated for DCIS die of something else. Yet even this small risk can be lowered with postsurgery radiation, which reduces the chances of subsequent invasive cancer by 50%, according to a 2011 study led by Irene Wapnir of Stanford University in California. Few people may try the experimental therapy when the norm looks so good. But Esserman and Hwang are running trials in which women diagnosed with DCIS opt out of some parts of standard therapy.

Dare to Do Less

Scientists are looking for ways to spare women from aggressive treatment of ductal carcinoma in situ, a diagnosis that only sometimes leads to invasive breast cancer

Shelley Hwang, a surgeon who has treated women with breast cancer for 17 years, is troubled by the thought that many who have gone under her scalpel really didn’t have cancer. What they had, she says, was irregular tissue that may increase the risk for cancer. Not knowing much about these abnormalities, however, oncologists decided decades ago that the right thing to do was to remove them. That’s still being done.

A tidal wave of such ambiguous cases began to pour into clinics in the early 1980s.

They were the product of a drive to catch cancer early, aided by new breast imaging methods that found lumps or tissue aberrations that would not have been noticed before. Hundreds of thousands of patients were told that they had “ductal carcinoma in situ,” or DCIS—cancer confined to a milk duct. It has also been called “stage zero” cancer. In the past, these women typically received mastectomies, followed by radiation and drugs.

Hwang, now at the Duke University School of Medicine in Durham, North Car-

To guide such treatment, Hwang says, “we need to have better predictors of which DCIS will likely become invasive and which won’t.” Progress has been slow, but a U.S. company—Genomic Health Inc. (GHI) of Redwood City, California—launched a test in December 2011 called DCIS Score, which monitors seven cancer genes in DCIS tumors to rate the risk that they will become invasive. Many physicians argue that its predictive value is small, and several university-based groups claim to have molecular markers that are better for detecting certain high-risk types of DCIS.

Still, both Hwang and Esserman describe the DCIS Score test as a useful first step. Such tools will help women decide which DCIS cases to wait and watch over, Esserman says. “We have to put just as much effort into making sure we don’t over-react” to the fear of cancer, she says, as we put into treating it.

Fear is the driver

“We didn’t have much DCIS in the United States until we got into mammography screening,” says Joann Elmore, an oncologist at the University of Washington, Seattle. Now, Elmore says, “we are seeing little calcifications, little white dots [on breast scan images]. We don’t want to miss anything,” especially because failure to detect cancer is “the number one cause” of malpractice allegations. When doctors do spot an anomaly, they are likely to ask for a biopsy.

For more than 60,000 women per year in the United States, that leads to a diagnosis of DCIS. The diagnosis is not new, but Hwang and others believe that the lesions now diagnosed as DCIS may differ from those with the same label in the 1940s and 1950s. They are smaller and, unlike earlier ones, most are not palpable. Under a microscope, Hwang says, the cells in the DCIS lesion look very similar to cells in an invasive breast cancer and are scored on three grades of abnormal appearance like those used for invasive cancer. But they are contained within the milk duct and may remain there safely for a lifetime. It’s

not known what enables some to escape. But it is clear that some do—and become dangerous.

The reported U.S. incidence of DCIS has increased dramatically over the past 3 decades, especially among women over 50. Estimates of the fraction of women diagnosed with DCIS who might go on to develop invasive cancer without treatment range from 14% to 50%. But because almost all cases are treated to prevent invasive cancer, it is difficult to get firm data. Pathological and molecular analyses of biopsies have already shown that some types of DCIS are more likely to be invasive, others far less, Esserman says. Yet even as doctors in the United States found and treated far more DCIS, the incidence of invasive breast cancer has remained fairly steady. To some, this suggests that treating all DCIS does not

“We were removing all these breasts” to take out DCIS lesions, “the majority of which might never become clinically significant.”

—Shelley Hwang,
Duke University School
of Medicine

do much to stop more dangerous cancers. This was the conclusion of a 2011 computer modeling study of cancer trends from 1978 to 2003 by Elissa Ozanne, then at Massachusetts General Hospital in Boston; Esserman; Hwang; and three others.

Forgoing the knife

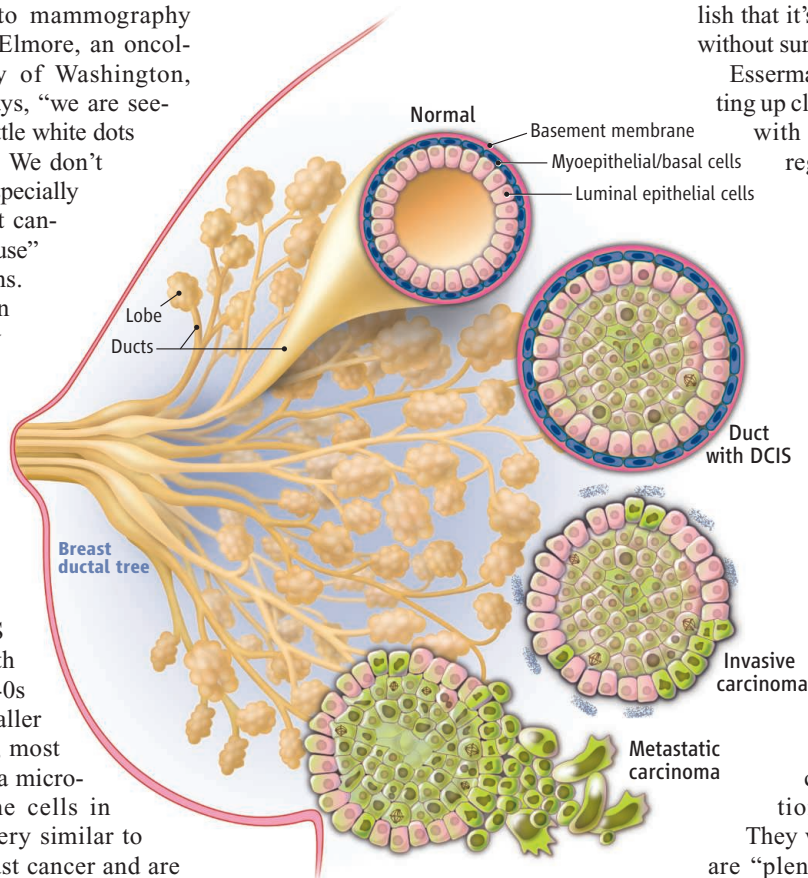
A few clinicians have begun to offer experimental ways of managing the disease. At Duke, for example, Hwang leads a cooperative trial at

23 U.S. sites that offers some women with DCIS an opportunity to try hormone therapy alone. Only postmenopausal women are enrolled, and only if their DCIS is rated low-risk based on traditional pathology measures and their lesions are estrogen-dependent. They are monitored closely to see if the DCIS starts to recede. Hwang hopes the trial, which ends in 2015, will establish that it’s possible to treat low-risk DCIS without surgery.

Esserman and colleagues at UC are setting up clinical trials to offer some women with DCIS a chance to enroll in a regime of “watchful waiting” and reduced treatment, similar to that offered to men with prostate cancer judged to be low-risk.

They rolled out a plan they call the Athena Breast Health Network in 2009, linking five UC medical centers. Esserman says the network is developing new classifications of breast lesions, using DCIS Score in combination with standard pathology to rate the chances that patients might develop invasive cancer. Patients judged to be high-risk will be treated as in the past, but others will be offered a choice of going without radiation, or even skipping surgery. They will be monitored closely. There

are “plenty of people” who are willing to live with some uncertainty, Esserman says, and “we should not browbeat them into having treatments” that may not benefit them.



Crossing the border. Tissue abnormalities known as DCIS may stay confined within a milk duct for a lifetime; a minority break out to become invasive cancer.

Testing times

As cancer physicians and their patients wait for results, women are left with little to guide them if they wish to avoid surgery, radiation, and drug treatments. Beyond the standard biopsy and personalized risk analysis based on genetics, they do have GHI's test, the only U.S. Food and Drug Administration-approved option on the market. Priced at \$4380, DCIS Score is a modified version of Oncotype DX, the test the company has sold for a decade as a way to classify the aggressiveness of invasive tumors.

DCIS Score probes tissue samples taken during breast biopsies for the activity of seven genes linked to cancer and five other reference genes. (Those genes include *Ki-67*, *STK15*, *Survivin*, *CCNB1* [cyclin B1], *MYBL2*, *PR*, and *GSTM1*.) Based on gene expression levels, an algorithm calculates whether a woman who has already had surgery (but not radiation) for DCIS is likely to see the lesion come back or even develop into invasive cancer.

Lawrence Solin, an oncologist at the Albert Einstein Medical Center in Philadelphia, Pennsylvania, led the first and, so far, only published effort to validate DCIS Score. With support from GHI, the U.S. government, and the Breast Cancer Research Foundation, Solin's group retrospectively analyzed tissue from 327 DCIS patients who had undergone surgery but not radiation. The team reported in 2013 that the three levels of risk scores the test gave for tissue samples correlated well with patient outcomes.

Women judged to have a low DCIS Score, for example, turned out to have only a 3.7% risk of developing invasive cancer within 10 years. Those with middling scores had a risk of 12.3%. And the highest scoring group had a risk of 19.2%. According to the authors, in their 327-patient sample, the gene test forecast risk better than current pathological methods, which rely mainly on the analysis of cell structure and physiology in biopsied tissue.

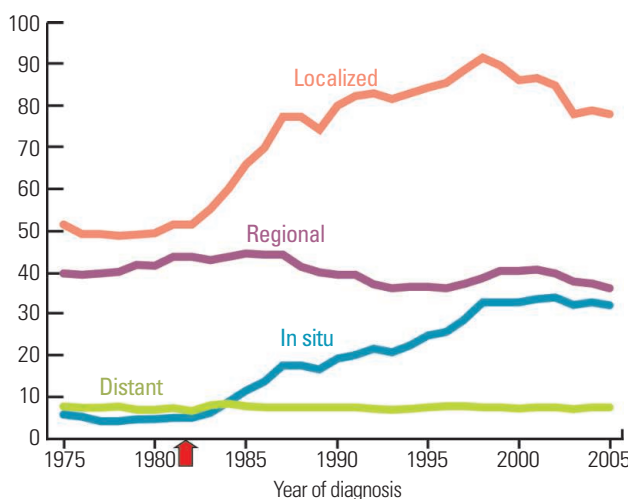
Many researchers aren't wowed by the test, however. Karla Kerlikowske, an oncologist and epidemiologist at UCSF, calls DCIS Score "OK," saying that at least it doesn't exaggerate risk. But she thinks that it doesn't flag some of the most dangerous

subtypes of DCIS, which are known to exist but are not fully defined. She and a group of researchers at UCSF have been analyzing additional potential markers of high-risk DCIS, which they would like to combine in a test. For example, they would include the activity of genes *p16* and *COX-2*, which are not in GHI's test.

Kerlikowske says the UCSF team has run comparative checks and found that DCIS Score "misses about half the invasive cancer" their method finds. The results are unpublished so far, however; Kerlikowske says she failed to win funding for a proposed full head-to-head comparison of the two assays. A company that was interested in the test 2 years ago has not yet moved ahead with it, she says.

U.S. Breast Cancer Incidence Rates 1973–2005

Per 100,000



Mammography's mixed impact. Breast screening surged in the 1980s (arrow), as did the detection of DCIS and localized cancers, but this didn't seem to reduce the worst, distant or metastasized, cancers.

Pathologist Agnieszka Witkiewicz and researcher Erik Knudsen of the University of Texas Southwestern Medical Center in Dallas have flagged a gene set that overlaps with UCSF's but also includes the well-known oncogenes *RB* and *PTEN*. "*RB* is probably our favorite," Knudsen says, because it gives more prognostic information in the group they've examined—DCIS patients from a Philadelphia clinic who had surgery but no radiation. He claims that this index "outperforms" DCIS Score in spotting the risk for certain types of invasive cancer. "If money were no object and I had a staff of thousands," Knudsen says, he would try to develop it.

Another DCIS risk assay still in the earliest stages of study focuses on protein levels in the duct's structure versus protein levels in the tumor. DCIS becomes invasive only when the duct gives way. In laboratory studies, Michael Allen and Louise Jones of the Barts Cancer Institute at Queen Mary University of London found that a protein known as integrin $\alpha\beta 6$, involved in cell signaling and attachment, was present at higher levels in the duct's myoepithelial cells when DCIS had become invasive. They report that this causes these ductal cells to change from tumor suppressors to tumor promoters. And they found a similar high level of $\alpha\beta 6$ in breast tissue from DCIS patients with invasive cancer. But Allen acknowledges that before these insights can be translated into a practical test, they must be tested in a clinical trial—and that would be difficult to carry off for many reasons.

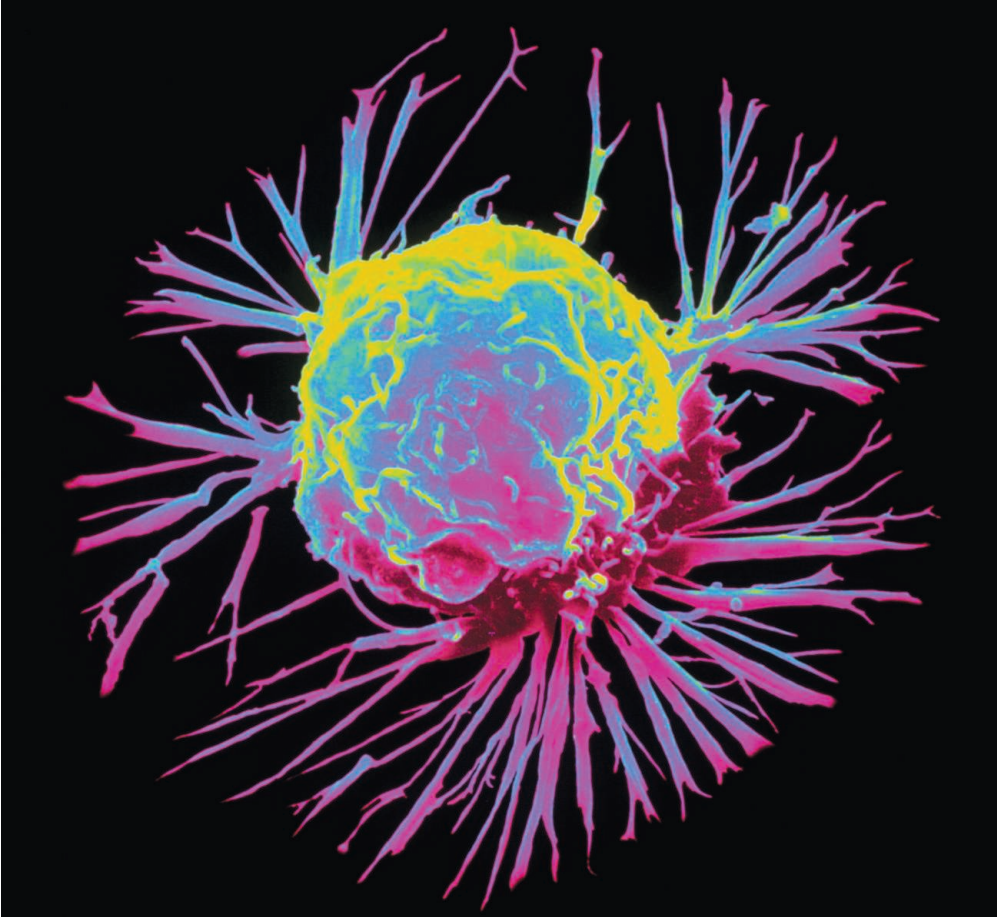
Until these would-be rivals standardize their methods and carry out trials, says Steven Shak, executive vice president of R&D and co-founder of GHI, their claims must be taken as interesting but not proven. Shak, who is a co-author on the Solin paper, claims that DCIS Score is "widely used." He adds that it is being validated in a second, independent patient group, although he declines to "upstage" the researcher who's running the trial by releasing details.

Competition for all the test designers may be on the horizon. The U.S. Department of Defense's Breast Cancer Research Program has proposed to aid the hunt for better DCIS risk markers. So has the U.S. National Cancer Institute, which plans to spend about \$5 million a year on such work, according to the director of its cancer prevention division, Barnett Kramer.

The help can't come fast enough, says Fran Visco, an attorney who heads the National Breast Cancer Coalition, an advocacy group in Washington, D.C. "We keep running down these roads putting a lot of time and money behind things but ... we don't have the basic information that we need." She gets calls from women "all the time" asking what they should do about a DCIS diagnosis. Visco tells them to study the data and balance the risks and benefits for themselves, because, "we don't really know the answer."

—ELIOT MARSHALL

GRAPH: ADAPTED FROM SEER DATA FROM OZANNE ET AL., BREAST CANCER RESEARCH AND TREATMENT (9 MARCH 2011)



The foe. What goes awry in a breast cancer cell (*left*) is becoming clearer, but scientists are still debating the role of different genes.

cancer genes has proved challenging. Geneticists simply don't know in many cases which mutations within a given gene will lead to cancer and which won't. A clearer picture will emerge only from long, involved trials to link mutations to clinical diagnoses.

This has led to a lot of hand-wringing among doctors and genetic counselors about what to do in the short term: when and whether to test the full panoply of other breast cancer genes in their patients, and how much to reveal to women who are tested. Some in the field even suggest not telling patients about certain mutations they harbor in their own DNA until geneticists can assess the risk more accurately, however long that takes.

Depending on what the tests turn up, most geneticists recommend that patients seek genetic counseling to help them understand the risks revealed by the tests. But not all women receive such counseling, especially in the United States, where testing companies can market directly to family physicians.

And even the best counseling can't alleviate the fundamental problem that the data continue to outpace the understanding. This has left doctors and patients alike in a bind, Ellis says. "You have to say ['I don't know'] a lot," he says, "and that's a pretty unsatisfactory answer."

Repair crew

Biologically, the role of the other breast cancer genes is becoming clearer. Like the *BRCAs*, most of them help repair broken DNA, says Mary-Claire King, who mapped *BRCA1* in 1990 and is a geneticist now at the University of Washington, Seattle.

Specifically, these genes help repair double-strand breaks. If a single strand of DNA's double helix breaks, cells can repair the flaw pretty easily by using the other, complementary strand as a template. Double-strand breaks are messier, and cells often have a tough time aligning the tattered fragments properly. Repair work therefore requires specialized machinery, and dozens of genes lend a hand.

If a woman inherits a mutation in one of

The 'Other' Breast Cancer Genes

Since the discovery of *BRCA1* and *BRCA2*, dozens more breast cancer genes have come to light. But what risk they pose—and what to tell women who carry them—remain quandaries

A quarter-century after the first breakthroughs in breast cancer genetics, scientists have a good grasp on the risks that come with mutations in *BRCA1* and *BRCA2*, the most prominent breast cancer genes. Frustratingly, though, just as the sky began clearing with the *BRCAs*, a whole flock of other genes has swept in and muddled the view.

BRCA1 and *BRCA2* dominated the breast cancer agenda for so long for good reason. *BRCA* cancers attack women early in life, they're aggressive and deadly, and they might hit three or four women in one family, a devastating legacy. On top of all that, they've been embroiled in political controversy, as the subject of a landmark Supreme Court case about the legality of patenting genes.

However infamous, though, the *BRCAs* account for only about 25% of breast cancers with a hereditary component. And with the rapid expansion of next-generation DNA sequencing, scientists have now unmasked dozens of other genes associated with breast cancer. *PALB2*, *ATM*, *CHEK2*, and others

probably won't gain the notoriety of either *BRCA* gene, but they've already become important players. "We always knew we should [test for] 10 genes instead of two," says Charles Perou, a geneticist at the University of North Carolina (UNC), Chapel Hill, "but it wasn't technically feasible in the past. Now it's practical."

In theory, women at high risk of breast cancer might benefit from such testing. They and any female relatives carrying a risky mutation could undergo screening more often, increasing the chances of catching tumors early. If the mutation looks especially risky, they might opt for prophylactic mastectomies, surgery to remove their ovaries, or sometimes both, because breast cancer and ovarian cancer share genetic risk factors. And if they do develop tumors, knowing the underlying genetic basis may help determine treatment.

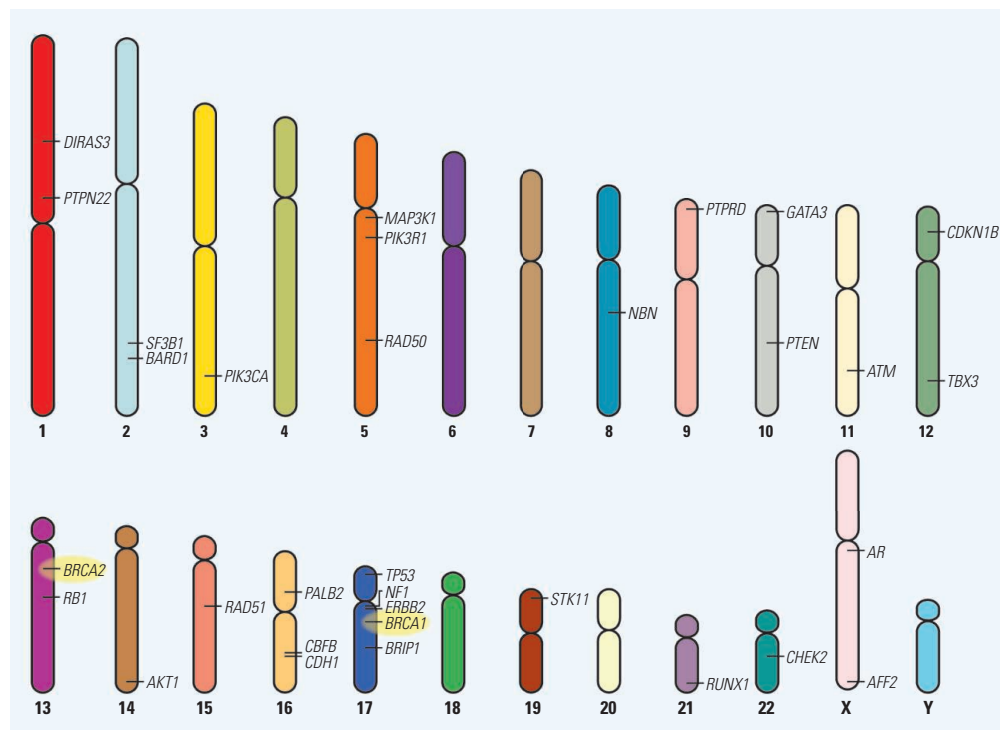
But as Matthew Ellis, a geneticist at Washington University in St. Louis, puts it: "As the genetic diagnosis gets easier, the interpretation gets more difficult." Identifying a risky mutation in these other breast

these genes, her cells can get along fine at first, because every cell has two copies of each gene, one on each chromosome. Problems arise only if the good copy gets disabled as well. Unfortunately, many genes involved in breast cancer have a high likelihood of getting shut down, King says, because they reside in “bad neighborhoods” on their chromosomes. For example, they might sit near a high concentration of *Alus*, mobile genetic elements that have a nasty habit of inserting themselves into genes and disrupting function.

If both copies of a DNA-repair gene end up disabled, the cell can lose the ability to repair double-strand breaks. At that point, King says, “all kinds of hell breaks loose” and breast cancer will likely result.

But geneticists still struggle to say which mutations in these new genes are important risk factors in cancer. Marc Tischkowitz, a geneticist at the University of Cambridge, sums up the difficulty succinctly: There is no *BRCA3*, he says. That is, no other single gene, when mutated, can explain a large number of breast cancer cases.

Consider *PALB2*, which interacts with *BRCA2* in repairing double-strand breaks. *PALB2* mutations cause a two- to threefold increased risk of cancer, a significant jump. (For comparison, harmful *BRCA1* mutations increase the risk of cancer by about fivefold; harmful *BRCA2* mutations increase it by



The “others.” Scattered across the chromosomes, dozens of genes (a sampling shown here) have now been implicated in hereditary breast cancer, but how much each contributes to a woman’s risk is unknown.

about fourfold.) But *PALB2* mutations appear so rarely that they probably don’t explain more than a tiny percentage of tumors. They’re so rare, in fact, that Tischkowitz had to organize an international consortium to study the gene, because no one country had enough cases. So while *PALB2* and other risky but rarely mutated genes contribute to our understanding of breast cancer, they explain few cases of breast cancer overall.

Adding to the difficulties, some mutations in the newly identified genes have incomplete penetrance. That means they only sometimes lead to cancer, making them tricky to interpret. If a woman has two or three low-penetrance mutations spread among a few different genes, scientists also don’t know in most cases whether the risks add up in a straightforward way.

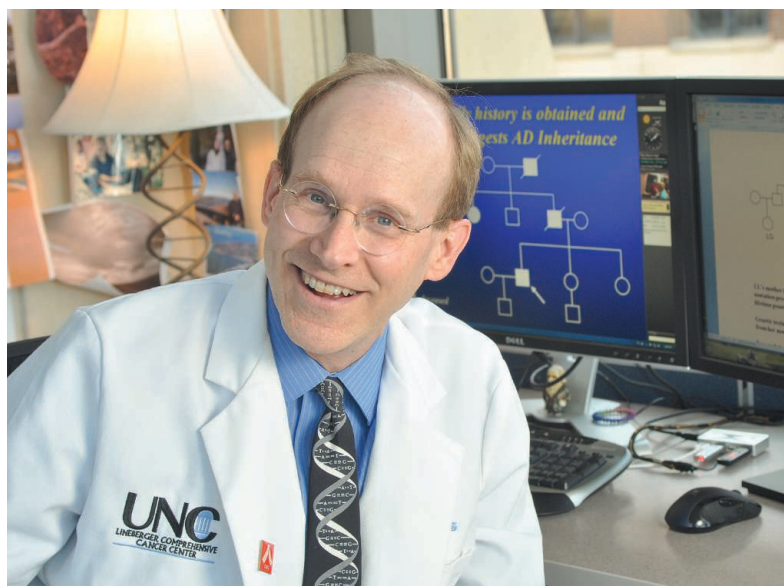
Some mutations are clearly worse than

others, King says. As examples, she mentions mutations that lead to premature stop codons during protein production, as well as insertions or deletions that lead to frameshift mutations. “Critical mutations” like these, she says, “whack the gene completely.”

Unfortunately, most mutations are more ambiguous. A point mutation near a functional domain, for instance, or a mutation in a noncoding region might well hamper a gene—but then again might not. Equally daunting, geneticists see huge numbers of different mutations in the population at large, each of which requires independent evaluation. In a phrase that

“We are now faced, daily, with decisions about whether to expand our reach in testing.”

—James Evans, University of North Carolina, Chapel Hill



pops up over and over—it's practically a mantra in breast cancer research—geneticists refer to these ambiguous mutations as “variants of uncertain significance.”

Scientists can now explain the genetic roots of about half of all family clusters of breast cancer, Tischkowitz says. “About a quarter of them will have mutations in *BRCA1* and -2, and a quarter of them will have a combination of common, lower penetrance variants. But about half of them are still unexplained. Which is a puzzle for us.”

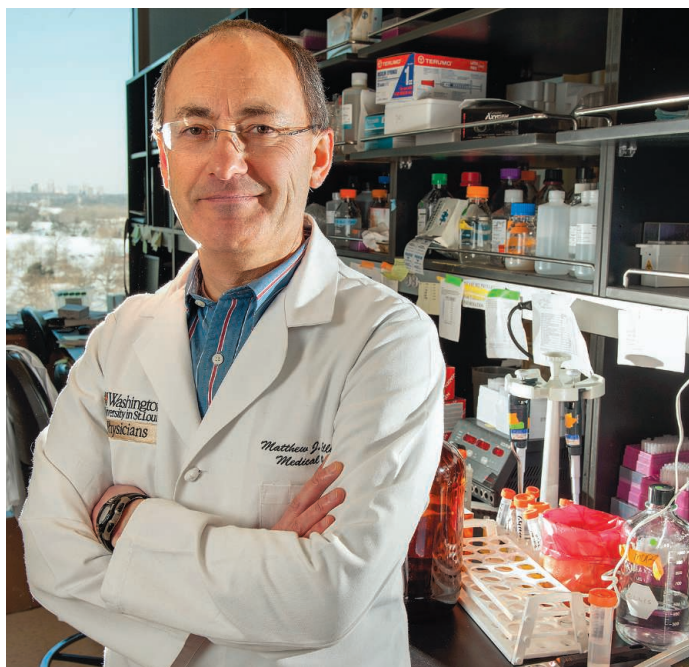
And things may get worse before they get better. Perou says that preliminary research has identified on the order of 100 other genes that might—or might not—also contribute to breast cancer. Sorting out the rogues from the pretenders, though, “is going to take some time,” he acknowledges. “It's much easier to make a discovery than to show that that discovery has clinical utility.”

In the clinic

Current tests for breast cancer reflect this uncertainty. “Six years ago, when we had our *BRCA1* and -2 results in hand, for most intents and purposes, we were done,” says James Evans, a professor of genetics and a genetic counselor at UNC Chapel Hill. But particularly in the past year, so-called expanded panels have come onto the market. These genetic tests scour for mutations in a dozen or more breast cancer genes. As a result, Evans says, especially with women whose families have a history of breast cancer, “we are now faced, daily, with decisions about whether to expand our reach in testing.”

Adding to the challenge, the panels differ from company to company, sometimes greatly, and the information they provide is not always helpful, he notes. He stresses that panels do have their use. “But the field has not settled on consistent, evidence-based guidelines,” he says, “that tell us when we should use an expanded panel, when we shouldn't, and even what genes should be on that expanded panel.”

To compound the problem, some genetic variants that predispose, say, Caucasian women to breast cancer might not predispose black or Hispanic women, and vice versa. So in addition to the general clinical trials necessary to link variants to an increased risk



“There’s a moral hazard here. Just adding genetic information doesn’t necessarily lead to better clinical management. A bad test can be as bad as a bad drug.”

—Matthew Ellis,
Washington University
in St. Louis

of cancer, scientists might also need to conduct separate clinical trials on different ethnic groups to sort out which mutations pose the worst risks for each.

Given all the uncertainty, geneticists and genetic counselors disagree about how often to use expanded panels, and especially about how much information they should pass along to patients. They do generally tell patients about unambiguously harmful mutations, and advise them to step up surveillance for early-stage tumors. But many geneticists hesitate to pass information about ambiguous mutations to patients. “I think it's probably better for them not to hear that,” Perou says. “It's confusing to me, it's confusing to them, and to be accurate, you would have to report a million variants for every person. That's completely not helpful.”

Ellis agrees. “There's a moral hazard here. Just adding genetic information doesn't necessarily lead to better clinical management.” He adds, “A bad test can be as bad as a bad drug.”

Faulty testing can also affect family members. In the 1990s, Tischkowitz says, preliminary studies strongly linked *CHEK2* to breast cancer; later studies reduced the risk substantially. But imagine that a geneticist had told a woman that a *CHEK2* mutation probably explained her family's history of cancer. Some of her sisters or daughters could easily test positive for the mutation as well.

In cases like this, Evans says, “you've just assigned a whole bunch of people in the family to screening and procedures that they didn't need.” Perhaps worse, other family members, who tested negative for the mutation, might believe themselves off the hook. “You've given them false reassurance,” Evans says, “and they haven't gotten the extra surveillance that they would benefit from.”

Above all, geneticists and counselors worry about inadvertently pushing patients to take drastic steps. “Patients will go to the end of the earth to protect themselves from breast cancer,” Ellis says, including prophylactic surgery. Mastectomies are still vastly more common among women with *BRCA* mutations, but a small percentage do elect for the surgery because of non-*BRCA* mutations.

So far, these cases seem limited to mutations with *PALB2* and other genes with rare but clearly harmful variants. Still, geneticists worry that, as more and more genes are linked to breast cancer, women might opt for mastectomies based on faulty or incomplete information, like the early *CHEK2* studies.

“One can imagine the headlines of people having unnecessary preventative surgery, when later on it's discovered that the risk of these genes may not be as strong,” Tischkowitz says. He adds, “Maybe we'll look back in time and think we were over-cautious, but I'd rather it be that way than the other way around.”

—SAM KEAN



The Advocate

For more than 2 decades, Fran Visco, president of the National Breast Cancer Coalition, has been a force behind the second biggest U.S. breast cancer research program

Frances Visco may be the most influential nonscientist ever in the field of breast cancer research. Her grassroots patient advocacy group, the National Breast Cancer Coalition (NBCC), has a small staff and does not run a research foundation. It has no scientific advisory board, although “we talk to scientists all the time,” says spokeswoman Michelle Zelsman. Yet the coalition has had an outsized influence on how breast cancer research money is spent. Twenty-two years ago, the group convinced Congress to put \$210 million into a new breast cancer research program in an unusual place—the Department of Defense (DOD). Since then, that program has awarded nearly \$3 billion in grants, making it the second largest funder of breast cancer research in the United States after the National Cancer Institute (NCI).

Visco has sat on the oversight board from the beginning. Her group has pushed for high-risk, high-reward research and studies that affect cancer patients’ lives. Many say the DOD program has been a big success, drawing new talent into the field and launching research that might not have made it through NCI review.

But Visco is not satisfied, noting that, in her group’s view, the decline in mortality from breast cancer over the past 2 decades is not significant. That’s why 4 years ago her coalition made another unusual move: It vowed to “end breast cancer by 2020” by attracting scientists to pursue what some say are wildly ambitious goals, such as a preventive cancer vaccine. For a group that prides itself on embracing evidence-based medicine, it was a surprising turn.

In a recent interview at the coalition’s office in downtown Washington, D.C., Visco discussed how its thinking has evolved since 1992. “In those days, we talked in terms of ‘We just need to find who the best scientists are, the most creative and make sure they have the money they need to do what they do,’” Visco said. “Maybe that was very naive.”

Call in the Army

Visco left a law practice to help launch NBCC after being diagnosed with breast cancer at age 39, joining other breast cancer activists including epidemiologist Kay Dickersin of Johns Hopkins University in Baltimore, Maryland, who had also had breast cancer, and breast surgeon Susan Love. They fol-

Call to action. Fran Visco took to the steps of the U.S. Capitol in 1997 to press Congress for continued funding for the Army’s Breast Cancer Research Program.

lowed the lead of AIDS activists, who had shown that patients could be a powerful force in boosting funding for their disease. In fall 1991, the newly formed coalition issued a call to supporters that drew 600,000 letters asking Congress to increase funding for breast cancer research. The group then held its own “research hearings” on Capitol Hill with handpicked scientists and concluded that to fund all promising ideas, NCI needed another \$300 million a year. Senators Tom Harkin (D-IA), chair of the Senate appropriations subcommittee that funds the National Institutes of Health (NIH), and Alfonse D’Amato (R-NY) agreed to boost NCI’s then-\$133 million budget for breast cancer research by \$64 million. They also put another \$210 million in the DOD budget, which already had a small women’s health research program, with the understanding that NCI would control how the money was spent.

But Visco’s group changed its mind after meeting with then-NCI Director Samuel Broder, who said “we can’t turn a huge battleship on a dime,” Visco recalls. She says her group had a “whole different experience” at the U.S. Army, where a general agreed the money should go to innovative research and that advocates should have a seat at the table. “We decided we wanted to keep it at the DOD,” Visco says.

To the relief of wary scientists, the Army asked the Institute of Medicine (IOM) for advice on how to spend the money—then expected to last just 2 years. The committee, which included Dickersin and virologist Harold Varmus, a Nobel Prize winner who soon became director of NIH, suggested that most of the money be spent on peer-reviewed basic research.

In a departure, IOM also recommended that the program’s steering committee, known as the Integration Panel, include advocates; the Army later added advocates as voting members of peer-review panels despite qualms from some scientists. The Integration Panel, the Army decided, would also have unusual discretion: Unlike institute councils at NIH, it doesn’t award grants based largely on peer-review scores but takes a “more hands-on” approach, Dickersin says. It can also take programmatic interests into account—for instance, it can reject a proposal with a stellar scientific score if it feels the program has already funded enough grants in that area.

Thanks to the coalition's lobbying, Congress has funded the DOD Breast Cancer Research Program every year since 1993, although not at the rate of growth of NCI, which now spends about \$600 million a year on breast cancer. The DOD program now receives between \$120 million and \$150 million a year. Nor are the grants easy to get: Success rates average 13% over 20 years, compared with 23% at NCI.

"A lot of people just look at it as another source of money," Dickersin says. But she and others argue that advocates trained in the peer-review process have pushed the program in a different direction than NCI. They add "a sense of urgency and passion as well as an important perspective on the feasibility and ultimate impact" of a proposal, says Gayle Vaday, a program manager with the DOD program. And because the DOD program has to spend all its money each year, unlike NCI, it can "move on a dime" and quickly launch new mechanisms or respond to new opportunities, says breast cancer researcher Dennis Slamon of the University of California, Los Angeles. In another review in 2004, IOM found "pretty strong consensus that DoD was adding value, not just replicating what NCI would otherwise do," says research professor of public policy Robert Cook-Deegan of Duke University in Durham, North Carolina, a member of that panel.

Many of the program's successful projects would have been unlikely to receive funding from NCI, researchers say. Slamon received one early grant to collect tumor tissue samples that firmed up his group's contention that some women's breast tumors carry extra copies of a gene coding for a surface protein, HER2, which makes the cancer particularly aggressive. NCI wasn't interested in funding the tissue bank because it wasn't hypothesis-driven, Slamon says. He adds that the stud-

ies helped persuade Genentech to move ahead with clinical trials of Herceptin, an antibody targeting HER2 that is now standard of care for this tumor type. Cold Spring Harbor Laboratory biochemist Gregory Hannon, a former chair of the Integration Panel, says the program supported his work using RNAi libraries to screen for genes that allow breast tumor cells to survive. The strategy was too "risky" to have a shot at NCI funding, Hannon says.

The program does have some detractors. Biostatistician Donald Berry, of the University of Texas MD Anderson Cancer Center in Houston, says that although he is "a big fan of Fran Visco and the NBCC" he thinks the DOD program would have benefited from input from a broader array of advocacy groups. The advocates on the Integration Panel tend to reflect Visco's and the coalition's views, he says.

Others see nothing amiss with the coalition's impact and Visco's long tenure on the Integration Panel. Visco's vote is just one of about 15, and she "embodies the guiding principles behind the program," Hannon says. "They [NBCC] have a lot of influence because the plain absolute truth is, there would not be this program without them," Slamon says.

If there is a shadow looming over the DOD breast cancer program, it is that each year

Added value. The U.S. Army's Breast Cancer Research Program, which has come on top of National Cancer Institute funding, has supported innovative research in a range of areas.

there is a chance that Congress will decide to kill it. With many champions having left Congress or planning to soon, including Harkin, Visco and her colleagues are working to cultivate new backers. One factor in their favor is that Congress over the years has expanded the program into a much larger Army peer-reviewed effort targeted for a range of diseases, from prostate cancer to prion disease, giving it broad appeal.

Agents of change

The National Breast Cancer Coalition now wants something more radical. Some 20 years and \$3 billion after the coalition's founding, "there hasn't been much in the way of real breakthroughs in breast cancer," Visco says. The incidence in the United States is still rising, and mortality is down only slightly, Visco argues. And there is too little research on prevention, she says. That led to the coalition's controversial new goal, announced in September 2010: to "end breast cancer by January 1, 2020." In an editorial on 29 November 2012, *Nature* called the 2020 goal "misguided" and wrote "[d]iscovery does not answer to deadlines."

Visco points out that the coalition has since emphasized that the specific goal is to "know how to end breast cancer by 2020." To do that, NBCC is encouraging research on two problems: how to prevent cancer with a vaccine and how to stop primary tumors from spreading, or metastasizing. These questions came from the advocates, not scientists, Visco says: "I think advocates can have a better idea than scientists because we're not just thinking about getting published or getting funded. We're thinking about what's best for women."

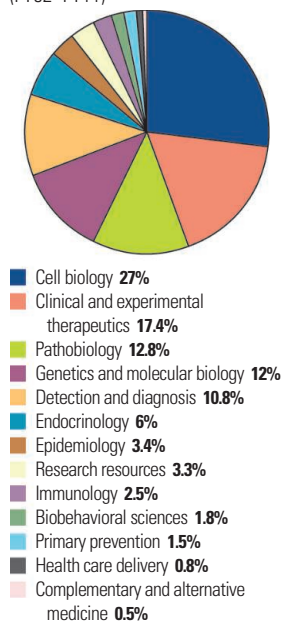
The coalition is using funding from a foundation to support seed grants for a few projects—chief among them genomics studies looking for antigens on breast cancer cells

or signs of a virus that could be used to design a preventive vaccine. Many breast cancer researchers have dismissed preventive vaccines as a long shot. And that's exactly why the coalition should encourage this research, Visco says. We're trying "to be disruptive and to change things. So this is our way of bringing back urgency to breast cancer."

—JOCELYN KAISER

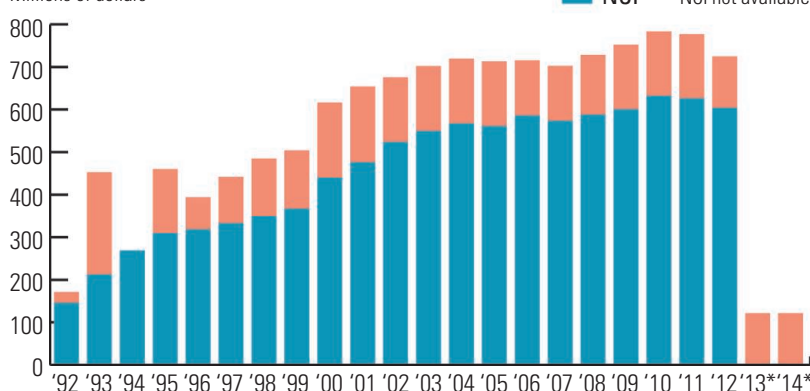
DOD Breast Cancer Portfolio

(FY92–FY11)



Annual Funding for Breast Cancer Research

Millions of dollars



PERSPECTIVE

"The Race" to Clone *BRCA1*

Mary-Claire King

The existence of *BRCA1* was proven in 1990 by mapping predisposition to young-onset breast cancer in families to chromosome 17q21. Knowing that such a gene existed and approximately where it lay triggered efforts by public and private groups to clone and sequence it. The press baptized the competition "the race" and reported on it in detail for the next 4 years. *BRCA1* was positionally cloned in September 1994. Twenty years later, I reflect on "the race" and its consequences for breast cancer prevention and treatment.

"An important part of the last few weeks has been ... to distinguish reality from fantasy. Fantasy has been 'the race' ... Reality is having the gene, not knowing what it does, and the realization that in the 20 years that we have been working on this project, more than 1 million women have died of breast cancer. We very much hope that something we do in the next 20 years will preclude another million women dying of the disease."

This comment is mine, from a late-breaking session of the American Society of Human Genetics (ASHG) in Montreal in October 1994. The session was devoted to *BRCA1*, whose sequence had been published in the 7 October issue of *Science* by a group led by Mark Skolnick, Sasha Kamb, and David Goldgar of Myriad Genetics (1). Their proof was the identification of mutations in their candidate gene for *BRCA1* that cosegregated with breast and ovarian cancer in five different families. As confirmation, our Berkeley group presented mutations in *BRCA1* cosegregating with breast and ovarian cancer in 10 additional families (2). From both groups, nearly all mutations led to predicted truncation of the protein. The function of the gene was completely unknown, but with 15 informative kindreds carrying mutations cosegregating with disease, the genetic evidence was indisputable.

That I was speaking in this session was thanks to Hunt Willard, chair of the program committee of the ASHG that year, who had made time on the program for a loser as well as for the winners. Now 20 years later, I am again grateful, this time to *Science* for highlighting the anniversary of the cloning of *BRCA1* and for inviting me to give a Perspective on "the race" to find the gene and the consequences of its discovery. Demonstrating the existence of *BRCA1* by linkage mapping had been the work of my small group at the University of California, Berkeley, from 1974 to 1990; "the race" to positionally clone the gene consumed the professional lives of more than 100 researchers

in at least a dozen labs for the next 4 years. Success required the exploitation of new genomic tools whose development advanced gene discovery for all illnesses and became the building blocks of modern genomics.

"The Race"

In 1990, after 17 years of work, my group mapped a hypothetical gene for inherited predisposition to early-onset breast cancer to chromosome 17q21 (3). The result was immediately confirmed by Gilbert Lenoir, Steven Narod, and their colleagues, who mapped predisposition to both breast and ovarian cancer to the same map location, with the same markers, in different families (4). The existence of a gene for predisposition to breast cancer, and the possibility of isolating it by positional cloning, triggered enormous interest in big labs in government, universities, and the private sector.

Linkage Analysis: The Back Story

I had come to the problem of inherited breast cancer by a circuitous route. After completing my dissertation at Berkeley, I moved to Santiago, Chile, to teach in the University of California–Universidad de Chile exchange program and then found myself jobless after the Chilean coup of 11 September 1973 led to the program's termination. My career in genetics, not to mention my mental health, was saved by Nicholas Petrakis of the University of California, San Francisco, who in January 1974 offered me a research position to study genetics of breast cancer, in whatever way I thought best. I started reading.

Descriptions of families severely affected with breast cancer date to ancient Greek physicians. In the mid-19th century, the early years of modern medicine, Paul Broca reported in detail on families with breast cancer in multiple generations. He postulated that breast cancer in these families was hereditary, present in a "latent state" until later in life when it presented and progressed in a malignant fashion (5). In the 1920s, Janet Elizabeth Lane-Clayton, a founder of modern epidemiology, demonstrated significantly greater mortality from breast cancer among women whose mothers had died of the disease compared with women whose

mothers had died of other causes (6). By the 1970s, multiple epidemiological studies demonstrated that the risk of breast cancer was increased in the daughters and sisters of affected women, particularly those with premenopausal or bilateral disease [e.g., (7)]. The absence of environmental exposures or lifestyle risk factors shared exclusively by affected women in these families was a consistent theme. Apart from their cancer predisposition, women in these families were remarkably healthy and productive. Such families seemed the perfect foundation for research.

Breast cancer is a common complex disease. Proving the existence of a causal gene required addressing these complexities, the most daunting of which was causal heterogeneity at several levels. Most cases of breast cancer have no inherited component, but given its high incidence, breast cancer with no inherited component will appear both sporadically and in multiply affected families. Conversely, multiply affected families may include both inherited and noninherited cases of the disease. Other complexities included penetrance of the hypothetical gene dependent on gender, on age, and on unknown nongenetic factors; the possibility of different genes responsible for predisposition in different families (locus heterogeneity); and the possibility of multiple different alleles at each of the hypothetical genes (allelic heterogeneity).

As a recent student of Allan Wilson, I thought about the problem in an evolutionary way. The impact of genes on any trait, including estimates of allele frequencies and effect sizes, could be modeled with the tools of population and evolutionary genetics. In principle, multiple different mechanisms for familial clustering of breast cancer were plausible, including influences of dominant or recessive major genes, polygenic effects, and/or shared environmental factors. Using complex segregation analyses to evaluate a sample of 1579 families ascertained through the population-based Surveillance, Epidemiology, and End Results Program of the National Cancer Institute, we demonstrated that familial clustering of breast cancer was fully explained by an autosomal dominant, highly penetrant susceptibility gene(s) (8). The maximum-likelihood model yielded estimates of the critical parameters: that 4% of families developed breast cancer due to a susceptibility gene; that among women carrying mutations in the gene, the risk of breast cancer by age 70 was 82%; and that among women without a susceptibility allele, risk of breast cancer by age 70 was 8%. The model was purely mathematical, and the gene was, of course, hypothetical.

The best way to demonstrate the existence of such a gene was to find it. Given the technology of the 1980s, "find" meant mapping a gene to a physical chromosomal locale using linkage analysis. Defining the breast cancer phenotype was most important. I was enormously lucky to have advice early on from Bernard Fisher, the father of

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minimal surgical treatment for breast cancer. "You're looking for the cause of invasive breast cancer," he said. "Don't get distracted." In taking his advice, we sought families including multiple relatives with invasive ductal carcinoma of the breast. In many of these families, breast cancer was early onset and often bilateral, and occasionally also appeared in men. We did not broaden the phenotype to include the far more common atypical hyperplasia, despite its being an established risk factor for breast cancer. Focusing on a narrow disease definition limited the number of persons in each family defined as "affected" and thus reduced the sample size for linkage, but far more importantly, it eliminated false positives. Keeping our eyes on the prize proved critical to successful mapping.

A rigorous definition of the phenotype did not solve other complexities, including causal heterogeneity and dependence of breast cancer expression on gender and age. To address these complexities, my colleague Ming Lee adapted the elegant linkage methods developed by Newton Morton, Robert Elston, and Jurg Ott (9) to incorporate the parameters of our previous population-based model into our calculations of the likelihoods of linkage of breast cancer to each of our genetic markers. To take an extreme example, an unaffected male or an unaffected female in her 20s did not contribute any information against linkage, whereas an affected male or an affected female in her 20s was highly informative.

Through the 1980s, with the help of oncologists, their patients, and the patients' families, we enrolled 23 extended kindreds severely affected with breast cancer. No detailed human genetic map yet existed, so gene hunters worldwide developed the genetic map collaboratively, in parallel with linkage analysis for each group's own projects. The first linkage markers were protein polymorphisms (10), then restriction fragment length polymorphisms (11), then variable number of tandem repeat (VNTR) markers (12), and—after the discovery of the polymerase chain reaction (13)—short sequence repeat markers (14). The development of the human genetic map had organizational homes at the Centre d'Etude du Polymorphisme Humain in Paris (15), led by Jean Dausset, Jean Weissenbach, Jean Marc Lalouel, and Mark Leppert; and with the beginning of the Human Genome Project, at the National Institutes of Health (NIH), led by Jim Watson. The human gene mapping period was characterized by open sharing of probes, DNA samples, and data, as well as by humor and good fellowship.

In our 23 extended families, Jeff Hall and I genotyped new markers as quickly as we learned of them or could develop them ourselves. Each marker was genotyped individually, the vast majority by Southern blot, in all informative persons.

The 173rd marker that we tested was *D17S74*, a highly polymorphic VNTR on chromosome 17q21. Linkage of breast cancer to this marker, using our model-based linkage parameters, yielded odds of 10^6 to 1 in favor of linkage for the seven families in our series with an average age of breast cancer onset ≤ 45 years and evidence against linkage for the 16 families with average age of breast cancer onset > 45 years. We published the results in 1990 (3).

"A path that began nicely enough with two or three adjacent cosmids would soon turn back on itself, yielding more a meander through a swamp than a walk from one signpost to another."

Positional Cloning

The Human Genome project was also born in 1990, so "the race" to find "the breast cancer gene," began with no genome sequence, no integrated physical maps, no awareness of genomic architecture, and certainly no genome browser. The mainstream estimate of the number of human genes was 100,000, based on inaccurate estimates of gene size and density. Very few genes were characterized and even fewer mapped. Sequencing was done by hand. There was no e-mail or Internet. The revolutionary advance in data sharing was the fax, with curly paper that found comfort hiding beneath your desk.

Following successful mapping, the process to gene discovery was positional cloning, which was experimentally challenging but fun. For *BRCA1* (which in 1991 I was allowed to name, because its existence was now widely accepted), we knew the chromosomal locale, defined by linkage and bounded by recombination at genetic markers in informative members of our families. The corresponding physical region was completely unknown. My group at Berkeley developed a collaboration with Francis Collins, then at the University of Michigan; with Anne Bowcock, then at the University of Texas Southwestern Medical Center; and later with Jeff Boyd, then at the National Institute of Environmental Health Sciences. Our joint strategy was to carry out four activities in parallel: continue genetic mapping with new markers to narrow the linkage region; generate a complete physical map of the linked region via a path of overlapping DNA-containing clones; hybridize the clones representing the region to a

cDNA library of genes expressed in our tissue of interest; and isolate and sequence these revealed genes from the cDNA library, then sequence each gene in DNA from affected members of our families to discover mutations disabling the gene.

Twenty years later, with the complete human genome sequence, it is now clear that the physical size of the region linked to *BRCA1* in our families was 22 Mb in 1990, 4.5 Mb by late 1992, and 1.0 Mb by early 1994, when all groups had resolved the critical recombination events in all families (16–18). Given the difficulty of physically mapping and cloning, the most productive period, by far, was after the region was reduced to a manageable size by linkage. Constructing the physical map was a tremendous challenge. At the time, DNA had been cloned into 40-kb cosmids by NIH and U.S. Department of Energy labs, and into much larger yeast artificial chromosomes (YACs) by Maynard Olson's lab (19). From their previous gene hunts, Francis Collins' lab was adept with chromosome walking with cosmids, but it was soon clear that the 17q21 region had many messy features that made these walks more difficult than previous ones. A path that began nicely enough with two

or three adjacent cosmids would soon turn back on itself, yielding more a meander through a swamp than a walk from one signpost to another. In retrospect, the problems were the high density of Alu sequences, of segmental duplications, and of pseudogenes in the *BRCA1* region. It is not surprising that a chromosomal region harboring a high-penetrance cancer susceptibility gene has so many bizarre architectural features. Odd genomic architecture predisposes to errors at mitosis and therefore to the somatic mutations that are the second hits of tumor suppressor genes.

For physical cloning, YACs were a cause for celebration because they captured much more DNA than did cosmids, so each tile (clone) was larger and the number of tiles needed to span a region was smaller. The corresponding challenges of YACs were that the longer the cloned insert, the more likely it was to be chimeric; that is, to include pieces of multiple chromosomes and to have internal deletion of elements of the chromosome of interest. The secret was not to be greedy, to work with YACs that were 100 to 200 kb—so longer than cosmids, but not grandiose. Bacterial artificial chromosomes (BACs) (20) had been developed at about the same time as YACs, but were not reduced to practice for human gene mapping for several years. BACs were intermediate in size between cosmids and YACs, so they are more stable than YACs and more informative than cosmids. BACs became a critical backbone of the Human Genome Project and, indeed, the Myriad group identified *BRCA1* from a BAC (1).

To keep the activities in our Berkeley, Ann Arbor, Dallas, and Research Triangle Park labs

straight, every day I wrote an "Order of the Day" (OOTD) that included progress with every cosmid, YAC, probe, library, genetic marker, and family. The OOTD was sent around by fax, then various small groups of people would chat by phone to plan the next experiments. This continued, if not 24/7, at least 14/6. The OOTD and the organization connected to it became even more important after Francis Collins moved from Michigan to the NIH in 1993 to take over direction of the Human Genome project from Jim Watson. With Francis' lab now in two places, we were four groups in five towns in three time zones, keeping in contact without Internet or cell phones. It seemed normal at the time.

The collaboration painstakingly identified hundreds of cosmids and YACs that mapped to our linked region. To maximize our success in capturing complete and critical genes, each group probed our shared clones to different cDNA libraries. In my lab, Eric Lynch created a beautiful cDNA library from the surface epithelial cells of an ovary donated by one of our participants at the time of her prophylactic surgery. We decided to create a cDNA library from ovarian epithelium rather than from breast epithelium, because breast epithelial cells are very difficult to dissect from other interspersed cell types. In contrast, ovarian epithelium covers the ovarian surface like a delicate blanket, so epithelial cells could be gently removed, the RNA isolated, and a library made of the expressed genes.

By hybridizing the cosmid and YAC clones from the linked region to this library, we iden-

tified more than 400 different cDNA clones and ultimately cloned and sequenced 15 genes from the 1-Mb region, as well as several others nearby (21, 22). Of these 15 genes, 5 were known but not previously mapped, and 10 were previously unknown. None carried loss-of-function mutations in our breast cancer families, because none was *BRCA1* (Fig. 1).

When the *BRCA1* sequence was published, how close were we to finding it? In retrospect, we had a marker inside *BRCA1* and did not know it. Our most informative linkage marker was AFM248yg9, in intron 20 of *BRCA1*, within a few kilobases of mutations in several of our families. Of course, we did not know this marker was inside *BRCA1*; we knew only that we could not identify a cosmid or YAC that carried it. In retrospect, cosmids carrying *BRCA1* were particularly difficult to identify because most of the intronic sequence of *BRCA1* is Alu. By masking Alu sequences to hybridize to the cosmid library, we masked the marker in *BRCA1* as well. Our physical map of the 1-Mb region had only one gap, exactly at *BRCA1* (Fig. 1). Of course, at the time, we had no idea of the size of the gap. Part of the plan was to create probes with single-copy DNA flanking each genetic marker and use them to find more clones. This would have revealed a cosmid containing *BRCA1*, then *BRCA1* itself. All groups in "the race" used essentially the same positional cloning strategy. The limiting factors were time and resources. As Maynard Olson remarked after visiting our lab, we were looking for

a pot of gold at the end of a rainbow in the backyard with a hoe, when what we needed was a backhoe.

The Gene

Even after it was cloned, *BRCA1* continued to confound its pursuers. For example, there is no exon 4 in *BRCA1*, because in the cDNA sequence published by the Myriad group, exon 4 was an Alu sequence with stops in all reading frames (1). The faux exon was soon gone from the sequence, but exon numbering did not change. More substantively, the genomic structure of *BRCA1* is distinctive, with a long central exon that encodes ~60% of the protein and is remarkably tolerant of amino acid substitutions. The 21 small flanking coding exons encode virtually all the functionally important regions of the protein. Likely for reasons of their parallel evolutionary histories, the genomic structure of *BRCA2* is almost identical to that of *BRCA1*, despite the two genes sharing no similarity in primary sequence (23).

At the time of its discovery, the biological function of *BRCA1* was completely unknown. There were no homologous genes and no recognizable motifs other than the RING domain (24), which was an acronym for "really interesting new gene," not a description of function. *BRCA1* could only have been found by a genetic approach. Because its function was unknown, it would not have been selected as a candidate gene based on a biological role. A genetic approach, however, is blissfully tolerant of total functional ignorance.

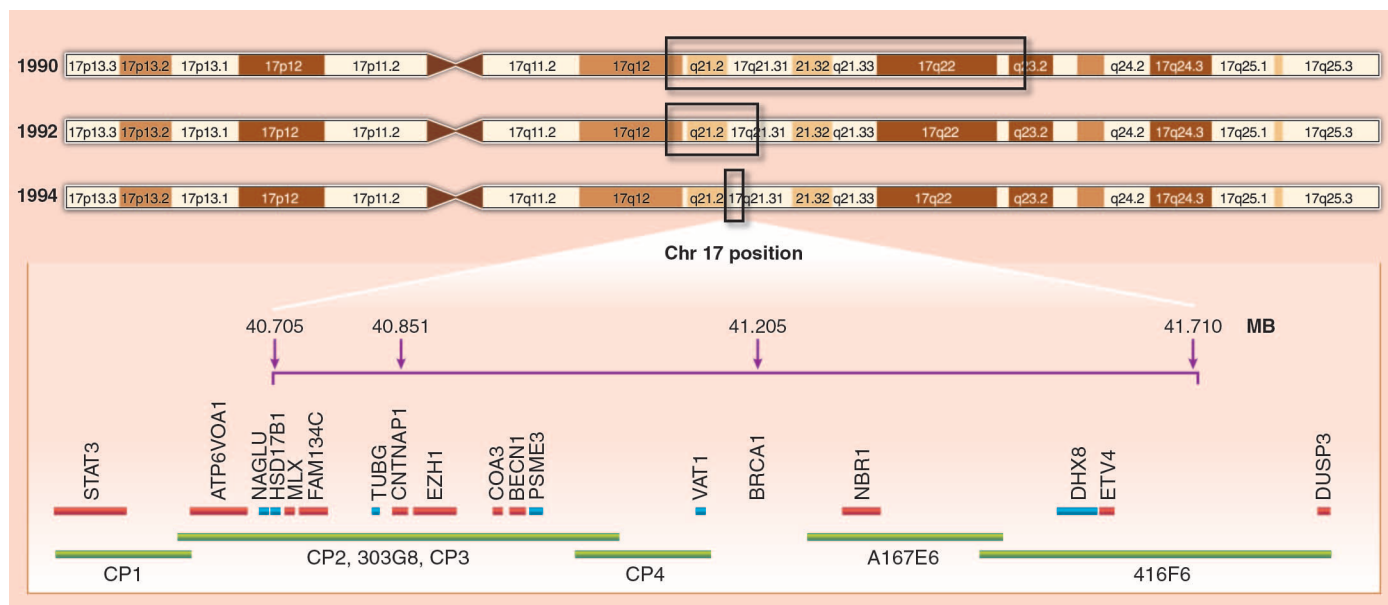


Fig. 1. Linkage and physical mapping of the *BRCA1* region as of early September 1994. Drawings of chromosome 17 at the top of the figure indicate refinement of the *BRCA1* region (black boxes) by linkage in 1990, 1992, and 1994. The final region defined by recombination in families was bounded by genetic markers (purple arrows), in retrospect known to lie at 40.705 and 41.710 Mb on 17q21.31, so yielding a linked region 1 Mb in length. This region was captured in YACs, P1 clones, and cosmid pools (CPs)

and assembled in physical contigs (green bars). In this 1-Mb region, we identified 15 genes, 5 previously known but not mapped (blue bars) and 10 previously not known (red bars). *BRCA1* was not captured because it lay in a 100-kb gap in the physical contig that was only fully sequenced 2 years later (37). In retrospect, the very dense packing of Alus in *BRCA1* led to it being refractory to clone capture. Thanks to the completion of the Human Genome Project, no gene hunter ever need face this problem again.

Twenty years after its discovery, the biological roles and evolutionary origins of *BRCA1* are still being elucidated. Genetics revealed that *BRCA1* is a tumor suppressor gene following the two-hit model (25): Cancer develops as the result of one inherited loss-of-function mutation followed by a somatic mutation causing loss of the remaining wild-type allele in a vulnerable cell type. The central puzzle is why complete loss of function of *BRCA1* leads to cancer. Solving this puzzle has been especially challenging, because the *BRCA1* protein is involved in multiple essential biological functions (26).

As part of a multiprotein complex, *BRCA1* repairs double-strand DNA breaks via the homologous recombination repair pathway. The C-terminal BRCT domain interacts with histone deacetylase complexes and is involved in transcriptional regulation. The N-terminal RING domain heterodimerizes with a sister domain of *BARD1* and acts as a ubiquitin ligase of the estrogen receptor (27). Missense mutations that abrogate the function of the RING domain lead to breast cancer. Virtually all other cancer-causing mutations of *BRCA1* are truncations: nonsense mutations, frame-shifts, or large genomic deletions or duplications leading to stops and loss of the C-terminal domain.

BRCA1 is ubiquitously expressed, so it has been a mystery why *BRCA1* mutations lead specifically to breast and ovarian cancer and, to a lesser degree, to pancreatic and prostate cancer. The estrogen receptor is a substrate of the ubiquitin ligase activity of the *BRCA1* RING domain, and missense mutations in critical residues of this domain lead to breast cancer predisposition (23). Very recent work indicates that estrogen controls the survival of *BRCA1*-deficient cells via a PI3K/NRF2-regulated pathway (28). *BRCA1* has revealed other breast cancer genes by virtue of the functional relationships of their encoded proteins. In particular, other genes critical for DNA repair—including *TP53*, *PALB2*, *CHEK2*, *BARD1*, *BRIP1*, *ATM*, *RAD51C*, and *RAD51D*—harbor mutations leading to inherited breast and ovarian cancer (29). Thousands of different disease-causing mutations have been detected in *BRCA1* and *BRCA2*. Each loss-of-function mutation is individually rare, and each independently confers very high risk for breast and ovarian cancer. The other breast and ovarian cancer genes also harbor many different rare, recent damaging mutations with effect sizes ranging from twofold increased risk for *CHEK2* to 10-fold for *TP53*.

Of the seven families in our 1990 linkage analysis with young-onset breast cancer (3), six families harbor mutations in *BRCA1*, and one harbors a mutation in *BRCA2*. Of the 16 families in that analysis that we predicted would not carry mutations in *BRCA1*, six are explained by *BRCA2*; one each is explained by *PALB2*, *CDH1*, and *SLX4*; and seven remain unsolved. There are more breast cancer genes to be found.

Genetic heterogeneity of inherited predisposition to breast cancer serves as a model for other

complex illnesses. The disorder results from any one of thousands of different mutations in any one of multiple genes. The critical genes known thus far encode proteins in the same and related pathways. The discovery of *BRCA1* and its sister genes illustrates that the degree of biological complexity underlying a phenotype is an excellent predictor of its genetic heterogeneity (30).

Our Genomes, Ourselves

In June 2013, the U.S. Supreme Court ruled unanimously that genes are products of nature and therefore cannot be patented (31), nullifying the Myriad patents on *BRCA1* and *BRCA2*. The ruling was a victory for science and for patients and led immediately to broader availability of clinical genetic testing.

For nearly 20 years, while Myriad was the only commercial source in the United States for genetic testing of *BRCA1* and *BRCA2*, cost was a major deterrent to widespread screening. The cost to women of *BRCA1* and *BRCA2* testing is now dropping, due both to the end of the monopoly and to two scientific developments that have changed the landscape. First, there are now enough genes identified with mutations predisposing to breast and ovarian cancer that multigene screening panels can be developed and effectively implemented. Second, genomic technology now offers the opportunity to sequence at costs orders of magnitude lower than the cost of Sanger sequencing (32). Previously, clinical genetic testing was carried out gene by gene, based on specific clinical indications and family histories, with each test costing thousands of dollars. With the advent of massively parallel sequencing, large panels of genes are now screened simultaneously at far lower cost (33).

There was another barrier to genetic testing for inherited breast and ovarian cancer. Some patients and physicians worried that a positive finding would lead to loss of health care coverage. In consequence, mutations were not identified in some women who could have been saved by risk-reducing surgery. Clinical guidelines have been established for women harboring damaging mutations in *BRCA1* and *BRCA2*, including increased surveillance, surgical removal of ovaries and fallopian tubes (salpingo-oophorectomy) by age 40 years or younger, and the possibility of risk-reducing mastectomy (34, 35). The Genetic Information Nondiscrimination Act of 2008 (Public Law 110-233), which protects mutation carriers against loss of health care coverage, should have removed fear as a barrier to testing, so that women with mutations in *BRCA1* and *BRCA2* can be identified without economic reprisal.

So what next? Given that 50% of *BRCA1* and *BRCA2* mutations are inherited from unaffected fathers, and given the small size of modern families, almost 50% of women with *BRCA1* and *BRCA2* mutations have little or no family history of breast or ovarian cancer. Yet, cancer risks to mutation carriers with no cancer family history

are as high as risks to mutation carriers from severely affected families (36). Identification of cancer-causing mutations in *BRCA1* and *BRCA2* has clear and actionable implications for prevention. *BRCA1* and *BRCA2* screening as part of routine health care for young adult women is sensible and feasible. As in any population-screening program, genetic or otherwise, few participants will prove positive, but for women who learn that they carry mutations in *BRCA1* or *BRCA2*, the consequences are enormous, addressable, and life-saving.

Until there are no more breast or ovarian cancers among women with *BRCA1* or *BRCA2* mutations, the real race is not over.

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PERSPECTIVE

Two Decades After *BRCA*: Setting Paradigms in Personalized Cancer Care and Prevention

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The cloning of the breast cancer susceptibility genes *BRCA1* and *BRCA2* nearly two decades ago helped set in motion an avalanche of research exploring how genomic information can be optimally applied to identify and clinically care for individuals with a high risk of developing cancer. Genetic testing for mutations in *BRCA1*, *BRCA2*, and other breast cancer susceptibility genes has since proved to be a valuable tool for determining eligibility for enhanced screening and prevention strategies, as well as for identifying patients most likely to benefit from a targeted therapy. Here, we discuss the landscape of inherited mutations and sequence variants in *BRCA1* and *BRCA2*, the complexities of determining disease risk when the pathogenicity of sequence variants is uncertain, and current strategies for clinical management of women who carry *BRCA1/2* mutations.

Up to 15% of patients diagnosed with invasive breast cancer have at least one first-degree female relative (mother, sister, or daughter) with the disease (1). A family history of breast cancer has long been thought to indicate the presence of inherited genetic events that predispose to this disease. Two decades ago, this association was confirmed when extensive studies of families with multiple cases of early-onset (<50 years of age) breast cancer led to the identification of two major breast cancer susceptibility genes, *BRCA1* and *BRCA2* (2–4). More than one million individuals now have been tested for mutations in *BRCA1* and *BRCA2*. Pathogenic mutations appear to account for ~30% of high-risk breast cancer families and explain ~15% of the breast cancer familial relative risk (the ratio of the risk of disease for a relative of an affected individual to that for the general population) (Fig. 1) (5–8).

Genetic testing for mutations in *BRCA1*, *BRCA2*, and other breast cancer susceptibility genes has served as a model for the integration of genomics into the practice of personalized medicine, with proven efficacy as a tool to determine eligibility for enhanced screening and prevention strategies, as well as a marker for targeted therapy. Here, we discuss the landscape of inherited mutations and sequence variants in *BRCA1* and *BRCA2*, the complexities of determining disease risk when the

pathogenicity of sequence variants is uncertain, and current strategies for clinical management of women who carry *BRCA1/2* mutations known to confer a high risk of breast and ovarian cancers. We also extend the discussion to consideration of the current clinical utility of genetic testing for mutations in other predisposition genes and common genetic variants that contribute to breast cancer risk.

Landscape of Mutations in *BRCA1* and *BRCA2* and the Cancer Risk They Confer

More than 1800 distinct rare variants—in the form of intronic changes, missense mutations, and small in-frame insertions and deletions—have been reported in *BRCA1* and 2000 in *BRCA2* (Breast Cancer Information Core; www.research.nhgri.nih.gov/bic). In *BRCA1*, missense mutations that are pathogenic and highly penetrant (i.e., confer a high risk of cancer) are located primarily in the RING finger and BRCT domains (2, 9, 10), which are critical for the DNA repair activity of *BRCA1*. In *BRCA2*, highly penetrant, pathogenic missense mutations are located predominantly in the DNA binding domain (11, 12). Large genomic rearrangements occur in both genes but are more prevalent in *BRCA1* (14% of mutations) than in *BRCA2* (2.6% of mutations) due to the large number of Alu repeats in the genomic region containing the *BRCA1* gene (13). Founder mutations (common mutations in a population arising from a small number of individuals) in *BRCA1* and *BRCA2* have been described in almost every population studied. The best known are in the Ashkenazi Jewish population, with 3% of individuals carrying one of the three founder mutations, namely *BRCA1* c.68_69delAG [185delAG] (1%), *BRCA1* c.5266dupC [5382insC] (0.13%), or *BRCA2* c.5946delT [6174delT] (1.52%) (14, 15). Other examples are the *BRCA1* c.548-?_4185+?del

[ex9-12del] mutation found in ~10% of Hispanic *BRCA* carriers and deletions of *BRCA1* seen in Dutch founder populations (16, 17). Thus, targeted screening for specific *BRCA1* and *BRCA2* mutations before full gene testing is warranted in a number of populations.

As studies of *BRCA1* and *BRCA2* unfolded, it became apparent that the estimates of penetrance (risk) of breast and ovarian cancer varied by the ascertainment criteria for studies, with population-based studies showing much lower risks than family-based studies (18). In clinical practice, *BRCA1* mutation carriers are generally estimated to have a 57% chance of developing breast cancer and a 40% chance of developing ovarian cancer by age 70, whereas *BRCA2* mutation carriers are estimated to have a 49% chance of breast cancer and an 18% chance of ovarian cancer (19). Interindividual variability in the risk of breast and ovarian cancer has been attributed to modifying environmental and genetic effects, including the location and type of mutations in *BRCA1* and *BRCA2*. Specifically, early reports focused on the location of mutations in *BRCA1/2* suggested that nonsense and frameshift mutations located in the central regions of either coding sequence, termed ovarian cancer cluster regions (OCCR), were associated with a greater risk of ovarian cancer than similar mutations in the proximal and distal regions of each gene (20–22). More recently, a Consortium of Investigators of Modifiers of *BRCA1/2* (CIMBA) study of 19,581 *BRCA1* and 11,900 *BRCA2* mutation carriers confirmed relative increases in ovarian cancer and decreases in breast cancer risk for mutations in the central region of each gene and higher risk of breast cancer for mutations in the 5' and 3' regions of each gene. Variability in risk is also partly explained by common genetic modifiers of breast and ovarian cancer risk in *BRCA1* and *BRCA2* mutation carriers that have been identified through genome-wide association studies (23–29). Accounting for these modifiers suggests that the *BRCA1* mutation carriers in the highest risk category may have an 81% or greater chance of breast cancer and a 63% or greater chance of ovarian cancer by age 80, whereas *BRCA2* mutation carriers at greatest risk may have more than an 83% chance of breast cancer by age 80 (27, 28). In conjunction with other variables modifying risk in *BRCA1* and *BRCA2* mutation carriers, these data offer the potential for more precise personalized risk estimates.

The Challenge of *BRCA1/2* Variants of Uncertain Significance and Variants That Confer Low to Moderate Cancer Risk

As described above, multiple mutations have been identified in *BRCA1/2* that inactivate the corresponding protein and increase the risk of cancer. However, many variants of uncertain significance (VUS), including missense, intronic, and small in-frame insertion/deletion variants, also have been observed. Although Myriad Genetics Laboratories

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has been able to classify many variants as neutral or pathogenic using proprietary data, other clinical testing laboratories offering *BRCA1* and *BRCA2* genetic testing cannot provide interpretation for many of the VUS encountered during testing due to limited information. In an effort to improve the classification process, the Clinvar (www.ncbi.nlm.nih.gov/clinvar) database has been posting results from some of the *BRCA1* and *BRCA2* clinical genetic testing conducted in the United States. Evaluation of VUS has often relied on error-prone models that predict the functional impact of variants on the basis of amino acid conservation and/or structure. However, the development of quantitative risk prediction methods by the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) has substantially improved assessment of the pathogenicity of VUS (30). This method estimates the probability of pathogenicity for each variant using combined evolutionary sequence conservation (Align-GVGD) (31), family-based segregation and cancer history, tumor pathology, and RNA splicing effects (12, 32, 33), and has resulted in classification of many *BRCA1* and *BRCA2* VUS as pathogenic or of neutral/low effect (33). Because this method often lacks statistical power due to the rarity of the individual VUS, quantitative cell-based in vitro assays that evaluate the effect of variants on established functions of the *BRCA1* and *BRCA2* proteins, with known sensitivity and specificity for pathogenic variants, have been de-

veloped for classification of *BRCA1* and *BRCA2* VUS (12, 34–36). Moving forward, interpretation of VUS pathogenicity will likely involve integration of functional, family, and pathology information in predictive models (37).

The classification of VUS may be further complicated by hypomorphic mutations in both *BRCA1* and *BRCA2*, which retain partial protein activity and may be associated with moderate to low risks of breast and ovarian cancer. The best characterized of these mutations is the p.Arg1699Gln (R1699Q) missense mutation in the BRCT domain of *BRCA1* that abrogates the repression of microRNA-155 (38) and is associated with a cumulative risk of breast cancer of 24% by age 70 (30). This risk is lower than that associated with other *BRCA1* mutations but substantially greater than the 12% risk of breast cancer in the general population. In contrast, the well-known polymorphic stop codon in *BRCA2*, p.Lys3326X, is associated with only a modest increase in breast cancer risk [odds ratio (OR) = 1.26] (39) and appears to have little clinical relevance. As more moderate risk variants in *BRCA1* and *BRCA2* are validated, risk management strategies distinct from those applied to carriers of high-risk mutations must be developed.

Clinical Management of Women Carrying Pathogenic *BRCA1/2* Mutations

Several general strategies can be used to reduce cancer risk, morbidity, and mortality in women

who carry pathogenic *BRCA1/2* mutations: (i) regular screening by imaging to detect tumors at an early stage; (ii) prophylactic surgeries—risk-reducing mastectomy (RRM) and/or risk-reducing salpingo-oophorectomy (RSO) (removal of the ovaries and fallopian tubes); and (iii) chemoprevention. In early studies, mammographic screening was found to have limited efficacy in detecting breast tumors in these high-risk women at an early, clinically actionable stage. Fully 29% of de novo tumors were missed by mammography but were found as a palpable mass after a normal screening examination, and a third of these tumors were detected when they had already metastasized to the lymph nodes (40). The limited success of mammography in this setting may result from difficulty in interpreting mammograms in young women with hereditary breast cancers who tend to have a higher breast density than older women, and because these hereditary cancers are often aggressive, rapidly growing “triple-negative” tumors (negative for estrogen and progesterone receptors and lacking HER2/neu amplification) (41). In contrast, magnetic resonance imaging (MRI) detects twice as many breast cancers in *BRCA1/2* mutation carriers as mammography or sonography (40), is associated with rates of interval cancers of less than 10%, and is now considered the standard of care. However, the increased sensitivity also results in increased false-positive rates, with 11% of women undergoing MRI with mammographic screening having

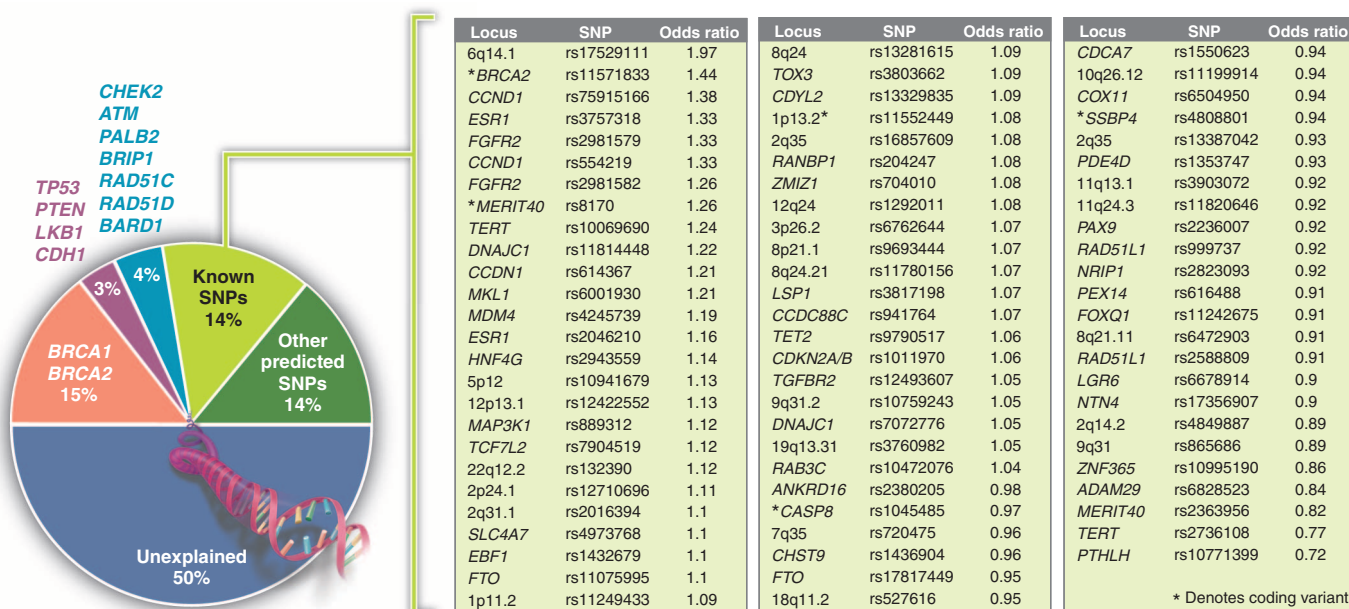


Fig. 1. Genetic variants that predispose to breast cancer. The pie chart on the left shows the estimated percentage contribution of mutations in high-penetrance (*BRCA1/2*, *TP53*, *CDH1*, *LKB1*, and *PTEN*) and moderate-penetrance (e.g., *CHEK2*, *ATM*, and *PALB2*) genes and common low-penetrance genetic variants to familial relative risk. Common genetic variants are denoted as SNPs.

“Known SNPs” are SNPs associated with breast cancer through GWAS, as listed on the right. The odds ratios refer to the increase (or, in some cases, the reduction) in risk conferred by the rare allele of the variants. “Other predicted SNPs” refers to the estimated contribution of all SNPs, other than known loci, that were selected for replication of breast cancer GWAS (5, 39).

biopsies that turned out to be benign, compared with 5% with mammographic screening alone (42–47).

Prophylactic surgical approaches are highly effective, with RRM reducing the risk of breast cancer by at least 90% in *BRCA1/2* mutation carriers (48, 49). However, due to the sensitivity of early detection using MRI, ~64% of women in the United States and 78% in Canada choose to avoid this surgery (50). In contrast, RRSO has become the standard of care for all women who carry highly penetrant *BRCA1/2* mutations because ovarian cancer screening methods using serum markers and imaging are largely ineffective (51, 52). RRSO has been shown to reduce the risk of *BRCA*-associated gynecologic cancer by 80 to 96% (53–55) and to reduce the risk of breast cancer by ~50%, most likely through the induction of premature menopause (54–56). Strikingly, RRSO has been shown to reduce the overall mortality of women by 60% with pathogenic *BRCA1* and *BRCA2* mutations (49). However, a 0.2% annual risk of cancer of the peritoneal lining around the ovaries and fallopian tubes remains because these tissues cannot be surgically removed by RRSO (53). Nonetheless, genetic testing for *BRCA1/2* mutations and RRSO provided an early example of the deployment of “personalized” prevention through genetics (40, 57).

Another clinical strategy found to reduce cancer risk in women with *BRCA1/2* mutations is hormonal chemoprevention. Antiestrogen therapy has been shown to decrease the risk of primary breast cancer in women at high risk who decided to retain their breast tissue, with several studies demonstrating up to 40 to 50% reduction in the risk of breast cancer in *BRCA1/2* mutation carriers taking antiestrogens such as tamoxifen (58, 59). Oral contraceptives also have been proposed as a strategy to decrease risk of cancer in women with intact ovaries, but with conflicting results. Some studies have shown a decrease in ovarian cancer risk in *BRCA1/2* mutation carriers by up to 60% with 3 or more years of oral contraceptive use (60, 61), whereas other studies have found a 30 and 50% increase in risk of breast cancer in *BRCA1* and *BRCA2* mutation carriers, respectively, with oral contraceptives use for 5 or more years (62, 63).

The identification of mutated genes that predispose to cancer often raises hope that understanding the biology of the corresponding proteins will lead to the development of new “targeted” therapies for patients. Establishing new paradigms in the application of genetics to personalized cancer care, the biology of *BRCA1* and *BRCA2* mutant tumors appears to be particularly well suited to specific therapies. In vitro and in vivo experiments and clinical trials have shown that platinum chemotherapy is effective against *BRCA1* (and, by analogy, *BRCA2*) mutant tumors, in part because platinum generates interstrand

cross-links that can only be adequately repaired by *BRCA1*- and *BRCA2*-dependent homologous recombination (HR) DNA repair (64). Mutations in *BRCA1/2* also sensitize cells to the inhibition of poly(ADP-ribose) polymerase (PARP), an enzyme involved in base excision repair (65, 66). Pharmacologic inhibition of PARP enzymatic activity in the background of *BRCA*-associated defects in HR-mediated DNA repair results in chromosomal instability, cell cycle arrest, and apoptosis. However, the exact mechanisms by which PARP inhibitors (PARPi) disrupt tumor growth remain to be fully delineated (67). Clinical trials have explored the efficacy of PARPi in the treatment of *BRCA1* and *BRCA2* mutant breast, ovarian, pancreatic, prostate, and other cancers, and it is likely that at least one of the four compounds entering phase II clinical trials this year will be licensed for widespread use (68). However, not all *BRCA* mutation carriers respond to these agents alone or in combination with chemotherapy. Indeed, studies with mice have suggested that mutations in the N-terminal BARD1 binding domain of *BRCA1*, such as the relatively common p.Cys61Gly (C61G), may not confer hypersensitivity to PARPi (69). In addition, as is the case with most targeted therapies, tumors can become resistant to these drugs (70, 71). Acquired resistance to PARPi has been associated with multiple mechanisms, including drug metabolism and efflux, post-transcriptional alterations of *BRCA1* or *BRCA2*, secondary mutations that restore the HR activity of *BRCA1* or *BRCA2*, and accumulation of somatic genetic alterations that counteract the sensitivity associated with *BRCA1* or *BRCA2* mutations (72). Whether combination therapies can overcome these complications remains to be determined.

Other Genes that Confer a Moderate to High Risk of Breast Cancer

Several rare cancer-susceptibility syndromes are known to confer a high risk of breast cancer, including Li-Fraumeni syndrome (caused by germline mutations in *TP53*), Cowden disease (caused by germline mutations in *PTEN*), and Peutz-Jeghers syndrome (caused by germline mutations in *STK11*) (Fig. 1) (73–75). Testing for mutations in these and other genes is part of the clinical management of women with a personal or family history suggestive of these diagnoses. With the advent of massively parallel sequencing and the ongoing delineation of an increasing number of genes mutated in familial breast cancer (for example, *PALB2*) (76), simultaneous screening of large panels of “predisposition” genes is now widely available. These panels have proven effective in identifying individuals and family members at elevated risk of breast and other cancers. However, clinical interpretation of results from the panels is complicated by several factors. In particular, breast cancer penetrance and risk of other cancers has not yet been established for pathogenic mutations

in most of the panel genes, and guidelines for clinical management of individuals found to carry these mutations have not been developed (77). Additionally, as is true for *BRCA1/2*, there is a high rate of VUS in the panel genes, the interpretation of which causes anxiety for both the patient and the physician. Furthermore, several commercial panels contain genes such as *APC* and *VHL*, which have not been clearly associated with susceptibility to breast cancer (78). Although continued clinical research is needed to responsibly integrate panel testing to practice, such approaches may provide guidance for critical clinical decisions such as whether a patient is at high risk of contralateral breast cancer and/or should undergo risk reduction surgeries. Conceivably, panel testing also may prove useful for selecting patients for treatment with PARP inhibitors, because several of the genes in current panels encode proteins involved in double-strand break repair, which may influence responsiveness to platinum and potentially PARPi (79).

Polygenic Risk Modeling

Genome-wide association studies (GWAS) of large numbers of breast cancer patients from the general population along with healthy controls have identified common genetic variants in 76 loci associated with small increases in the risk of breast cancer (Fig. 1) (39, 80). The greatest influence on overall breast cancer risk identified through GWAS is associated with the rs35054928 variant in the *Fibroblast Growth Factor Receptor 2* gene (*FGFR2*) (OR = 1.27) (81). However, many of the other variants have minor effects on risk (OR < 1.10) (39). The majority of the known variants are associated with estrogen receptor (ER)-positive breast cancer, but seven loci are specific to ER-negative disease (82). Little is known about the relevance of these risk factors to the different molecular subtypes of breast cancer, although three of these loci (*MDM4*, 19p13.1, and *TERT-CLPTM1L* rs10069690) are exclusive to triple-negative breast cancer (82–85) and *BRCA1*-associated breast cancer (27). Several of the common breast cancer risk variants are associated with established cancer genes such as *BRCA2*, *TGFBR2*, *MYC*, and *TET2* (39), but the underlying biological mechanisms by which most of these common variants influence breast cancer risk are not well understood. Recent evidence suggests that many of these risk loci contain multiple independent risk-associated variants that may have combined effects on gene transcription. For instance, two variants in the 11q31.1 locus with independent effects on breast cancer risk regulate Cyclin D1 expression by modifying a transcriptional enhancer and a silencer of the *CCND1* gene (86). Similarly, two independent risk-associated single-nucleotide polymorphisms (SNPs) in the *FGFR2* locus induce FOXA1, ER α , and E2F1 binding to enhancers and promote FGFR2 expression (81). Extensive fine-mapping and functional

studies are needed to determine how common genetic variants increase breast cancer risk in the general population.

Documentation of the clinical utility of risk-associated SNPs constitutes a key hurdle in the emerging paradigm of polygenic risk assessment for human cancer (84–86). The first such effort for breast cancer showed that 10 breast cancer-associated SNPs, when combined with traditional breast cancer risk markers, had a modest impact on risk prediction models (87). A subsequent study indicated that 15 SNPs added little to discriminatory accuracy but did reclassify 8% to 32% of women for MRI eligibility and 11% to 19% for tamoxifen use (88). In addition, a polygenic risk score (PRS), including 22 SNPs, calculated as the sum of the ORs for each allele, correlated with risk of early onset breast cancer (OR = 3.37, $P = 0.03$) (88). Several studies examining the influence of all known breast cancer-associated SNPs on risk are now under way (85). Overall, it now appears likely that combinations of risk variants will improve stratification of the risk for breast cancer, leading to better identification of women who will benefit from enhanced screening and intervention (89).

Conclusions

The clinical management of breast cancer is continually evolving to incorporate new information emerging from studies of the basic biology of the disease. History provides many examples: the progression of surgical approaches from the Halstead radical mastectomy to sentinel node sampling, the incorporation of gene expression microarrays to subclassify the disease and serve as prognostic biomarkers, and the early development of a targeted therapy (Herceptin) for breast cancers overexpressing the HER2/neu receptor. The role of PARP inhibitors for treatment of breast cancers with *BRCA* mutations has established a new paradigm of targeted therapeutics directed toward an inherited genetic susceptibility. Similarly, the elucidation of the drivers of hereditary breast cancer, characterized by gene-gene and gene-environment interactions of rare mutations and common variants, exemplifies an emerging model of the polygenic basis of this common human malignancy (5–7, 57, 80, 90). As part of personalizing risk assessment, these genomic insights may soon form a rational and cost-effective basis for selection of women for breast cancer screening (91, 92). Going forward, the reduced cost and increased access to genomic profiling of breast tumors will likely identify new therapeutic targets. However, the anticipated increased uptake of sequencing will require new approaches for communication to patients of findings from germline DNA that suggest increased risk for treatment toxicities or risk for disorders other than breast cancer (90, 93, 94). Two decades after the cloning of the *BRCA* genes, clinical application of findings of breast cancer genetic research continues to drive new para-

digms of “personalized” genomics and precision medicine.

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PERSPECTIVE

Cancer Suppression by the Chromosome Custodians, BRCA1 and BRCA2

Ashok R. Venkitaraman

Germline mutations in *BRCA1* and *BRCA2* predispose to common human malignancies, most notably tumors of the breast and ovaries. The proteins encoded by these genes have been implicated in a plethora of biochemical interactions and biological functions, confounding attempts to coherently explain how their inactivation promotes carcinogenesis. Here, I argue that tumor suppression by *BRCA1* and *BRCA2* originates from their fundamental role in controlling the assembly and activity of macromolecular complexes that monitor chromosome duplication, maintenance, and segregation across the cell cycle. A tumor-suppressive role for the BRCA proteins as “chromosome custodians” helps to explain the clinical features of cancer susceptibility after their inactivation, provides foundations for the rational therapy of BRCA-deficient cancers, and offers general insights into the mechanisms opposing early steps in human carcinogenesis.

The landmark discovery that germline mutations affecting *BRCA1* or *BRCA2* trigger inherited susceptibility at high penetrance to cancers of the breast and other organs sparked intensive investigations into the mechanisms by which their protein products, localized primarily in the cell nucleus, work as cancer suppressors. Over the past 20 years, however, these studies have unearthed many physical and functional connections made by the BRCA proteins in diverse biological processes whose links to cancer pathogenesis remain uncertain. I argue here that the role of *BRCA1* and *BRCA2* as custodians of the structural and numerical integrity of chromosomes during the cell cycle underlies their tumor-suppressive function. I will discuss how this conceptual framework helps to explain the clinical features of cancer susceptibility in *BRCA* mutation carriers, reveals principles underlying new approaches for treatment, and offers a powerful experimental paradigm for understanding how chromosomal instability (1) contributes to human carcinogenesis.

Tumor Suppression and the Functions of BRCA1 and BRCA2

Discovery of an Essential Role in Chromosome Integrity

Key studies in the first few years after the discovery of *BRCA1* and *BRCA2*, which defined their essential function in preserving chromosome integrity during cell division, have been instrumental in guiding subsequent work. Targeted dis-

ruption of both copies of *BRCA1* or *BRCA2* in the mouse germ line was shown to provoke early embryonal lethality and impede cell proliferation (2–7). This is accompanied by hypersensitivity to genotoxins (4–6, 8), consistent with the migration of *BRCA1* and *BRCA2* proteins (Fig. 1) to nuclear foci triggered by DNA damage (9, 10), and their interaction with different proteins implicated in the cellular response to such lesions (4, 11–13). It is remarkable that *BRCA2*-deficient cells spontaneously accumulate aberrations in chromosome structure and number during division (6). The structural aberrations typically include breaks affecting a single sister chromatid, as well as quadriradial and triradial chromosomes. Both types of abnormality signify defects in homologous DNA recombination and are also characteristic of two other cancer susceptibility syndromes, Bloom syndrome and Fanconi anemia (6). *BRCA2*-deficient cells exhibit translocations, large deletions, or fusions that involve multiple, nonhomologous chromosomes (14). These structural anomalies are accompanied by aberrations in chromosome number reflecting inaccurate chromosome segregation (6). Cells lacking *BRCA1* exhibit similar defects (15). Collectively, these findings establish that *BRCA1* and *BRCA2* act as custodians of chromosome integrity during the cell cycle, in turn engendering a model (16, 17) wherein *BRCA* inactivation fosters carcinogenesis by promoting chromosomal instability.

Protein “Hubs” Protecting Chromosome Integrity

The precise mechanisms by which *BRCA1* and *BRCA2* protect chromosome integrity during the cell cycle remain unclear. Notable confounding

factors include the number and diversity of proteins that have been reported to physically interact with *BRCA1* and *BRCA2* (fig. S1), the localization of *BRCA1* and *BRCA2* to different intracellular compartments and structures during the cell cycle, and the shifting nature of these properties in response to cellular signals that trigger posttranslational modifications, such as phosphorylation or ubiquitylation. These features suggest that *BRCA1* and *BRCA2* may belong to a small subset of proteins that serve as dynamic “hubs” for multiple macromolecular complexes. Hub proteins typically contain one or more intrinsically disordered regions, which lack a defined three-dimensional structure in isolation but, instead, tend to acquire more stable conformations when they bind to other macromolecules (18). For instance, the 1863-residue human *BRCA1* protein encodes a structured RING domain at its extreme amino (N) terminus and tandem BRCT domains at its carboxyl (C) terminus, but the long central region between residues 170 and 1649 is predicted to exhibit intrinsic disorder by *in silico* and experimental results (www.disprot.org) (19). Such analyses also suggest that the 3418-residue human *BRCA2* protein likewise contains intrinsically disordered regions dispersed between more structured segments (www.disprot.org) (20, 21). Some of these structured segments or motifs (for example, the RING or BRCT domains in *BRCA1* and the BRC repeats and OB folds in *BRCA2*) (Fig. 1) also occur in proteins from simpler organisms with overlapping functions. However, these simpler proteins are typically smaller and less complex, and their functions are likely more limited than those of the corresponding BRCA protein, as illustrated by the *BRCA2* ortholog Brh2 from *Ustilago maydis* (22).

These considerations suggest that the large *BRCA1* and *BRCA2* proteins act as segmental entities, in which distinct and sometimes intrinsically disordered regions enter into different physical interactions to perform distinct biological functions. In this way, the BRCA proteins may subsume and coordinate across the cell cycle the work performed by multiple protein complexes in simpler organisms. Not all of these functions are necessarily relevant to tumor suppression. Moreover, overexpression or cell-free biochemical studies on hub proteins like *BRCA1* and *BRCA2* may elicit false clues owing to the likelihood of promiscuous interactivity in such experimental settings. From this perspective, the phenotypes provoked by *BRCA1* or *BRCA2* deficiency in cells, model organisms, and patients provide a

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valuable yardstick against which to measure functional understanding.

Distinct BRCA1 and BRCA2 Functions Prevent Chromosome Structural Aberrations

The characteristic structural aberrations that accumulate spontaneously during the division of BRCA-deficient cells focus attention on double-strand DNA breaks (DSBs) or daughter-strand gaps (DSGs) created during chromosome duplication [reviewed in (23)]. DNA replication forks frequently stall at lesions in the template strands even during normal cell proliferation. Homologous DNA recombination (HR)—a mechanism for the error-

free repair of DNA breakage by exchange with an intact, homologous sequence (such as the sister chromatid)—is central to the resolution of stalled forks. Stalled forks may be cleaved to generate a DSB that can be repaired by HR. Alternatively, a replication bypass around the arrested fork, leaving behind the arrest-inducing lesion, may create a DSG that can be resolved by HR without a DSB intermediate. BRCA1 and BRCA2 are individually essential for efficient HR in mammalian cells (24–28). In their absence, replication-associated DNA breaks can instead be repaired by error-prone mechanisms like nonhomologous end joining (NHEJ) and microhomology-mediated

end joining (6, 14, 26–28), which promiscuously re-ligate broken ends, particularly across short microhomologies. Thus, BRCA deficiency reroutes replication-associated DSB or DSG repair down an error-prone pathway. Consistent with this notion, human cancers harboring homozygous mutations in BRCA1 or BRCA2 display not only extensive structural rearrangements in chromosomes but also many short deletions (≤ 50 bp) with overlapping microhomology at breakpoint junctions (29).

BRCA1 and BRCA2 have distinct functions that ensure the error-free resolution of replication-associated DNA damage (Fig. 1), although their

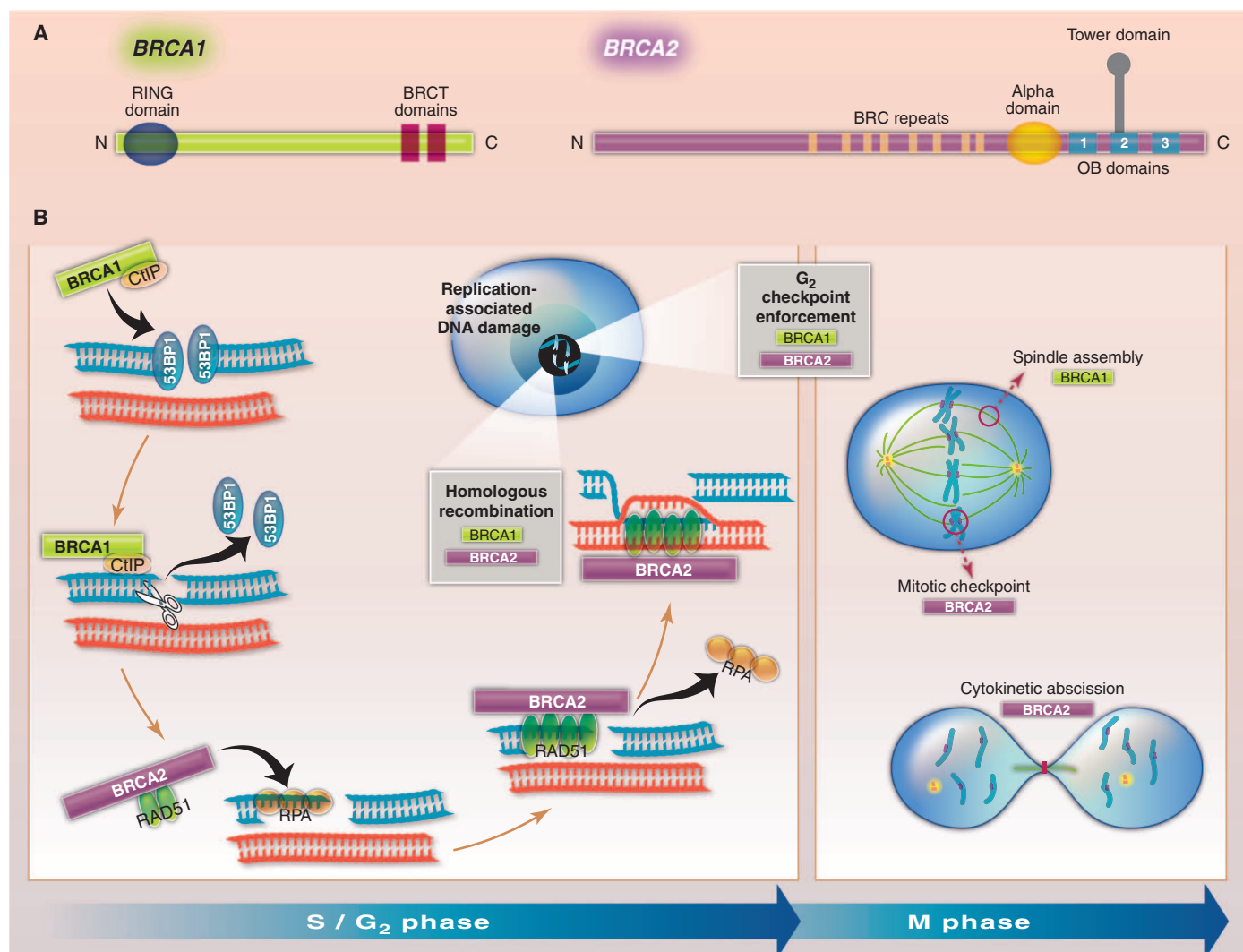


Fig. 1. BRCA1 and BRCA2: chromosome custodians. (A) The structural domains of human BRCA1 (1863 amino acids) and BRCA2 (3418 amino acids) proteins. An additional RAD51-binding region at the C terminus of BRCA2 is not marked, for simplicity. (B) Functions of BRCA1 and BRCA2 at different stages of the cell cycle discussed in the main text. During HR-mediated repair of replication-associated DNA damage, BRCA1 helps to initiate HR by displacing 53BP1 (p53 binding protein 1), thus triggering end resection (scissors). The C-terminal DNA binding domain of BRCA2 binds to the junction between ssDNA and dsDNA at the lesion, displacing RPA, an ssDNA-binding

protein. The BRC repeats of BRCA2 bind to RAD51 recombinase and recruit it to the lesion. The BRC repeats promote nucleoprotein filament formation by stabilizing RAD51-ssDNA interactions while inhibiting RAD51-dsDNA binding. Both BRCA1 and BRCA2 participate in G₂ checkpoint enforcement. During mitosis, they promote mitotic spindle assembly (BRCA1) and regulate the mitotic checkpoint (BRCA2). BRCA2 participates in the abscission step of cytokinesis through functions at the midbody. Not all proposed functions of the BRCA proteins in chromosome maintenance are shown. Further detail is provided in the text.

partner protein PALB2 (30) bridges formation of a BRCA1-BRCA2 complex that assists in their localization there (31, 32). A simplified view is that BRCA1 primarily acts at proximal steps that signal the presence of these lesions and helps initiate their repair by HR, whereas BRCA2 stabilizes the structure of replication-associated lesions and works directly to resolve them using HR, by controlling the activity and assembly of the essential recombination enzyme RAD51. Many studies not discussed here that add biochemical detail to this simplified view are more relevant to our understanding of HR than of tumor suppression by BRCA1 or BRCA2.

The C-terminal tandem BRCT domains of BRCA1 recruit it to damage sites. Initial recruitment likely proceeds via their ability to bind poly (ADP) ribose (PAR) chains conjugated to proteins at these sites (33), whereas recognition of phosphopeptide motifs with the consensus (pSer) XXpHe, created when protein kinases that signal DNA damage become activated, promotes continuing accumulation. Thus, BRCA1 BRCT domains engage phosphorylated Abraxas, a protein that bridges BRCA1 complex formation with RAP80, a protein that binds to ubiquitin conjugates at damage sites via multiple ubiquitin-interacting motifs (34–36). The BRCT domains also bind to the BRIP1 DNA helicase mutated in Fanconi anemia (37) and the CtIP protein (38), interactions that are mutually exclusive between themselves and with Abraxas (34, 35). These findings suggest that BRCA1 uses the BRCT domains to manage the assembly and activity of several distinct macromolecular complexes (39) and performs varied functions at sites of DNA damage or replication stalling.

For HR to initiate, flush-ended broken DNA must be resected by a 5' to 3' nucleolytic activity to expose 3'-overhanging single-stranded (ss)DNA tracts that become the substrate for HR reactions. Regulation of this step by BRCA1 may help to restrict HR to the postreplicative G₂ phase of the cell cycle, during which a duplicated sister chromatid is available to template the repair of its damaged partner (40–43). HR is normally suppressed during the G₁ phase when the 53BP1 protein binds to broken DNA ends and recruits cofactors to prevent end resection. During G₂, a macromolecular complex containing BRCA1 displaces 53BP1 by an undefined mechanism, which facilitates end resection to initiate HR (Fig. 1). Thus, BRCA1 may help route the repair of replication-associated DNA damage via error-free HR, by displacing the HR-suppressing factor 53BP1 and its partners from broken ends. Supporting this view, 53BP1 inactivation partly reverses the structural chromosome aberrations induced by BRCA1 deficiency (40, 41). How BRCA1 displaces 53BP1 to initiate HR remains controversial. CtIP binding via the BRCT domains has been implicated (43) and, in addition, recently was suggested to regulate, in competition with 53BP1, the extent

of genetic exchange during later steps in HR (44). However, the recent demonstration that the BRCA1-CtIP interaction is dispensable for resection-mediated HR, DNA damage responses, and tumor suppression in transgenic mice (45) raises questions about its functional significance.

After end resection, the abundant ssDNA-binding protein RPA (replication protein A) coats exposed single-stranded (ss) DNA at chromosomal DSBs. RPA must be displaced to allow HR to proceed. This function is performed by an ~800-residue segment within BRCA2, which becomes ordered upon binding to the small acidic protein DSS1, and forms a protein complex that contains repeated oligonucleotide-oligosaccharide-binding (OB) folds capable of binding to ssDNA,

in addition to a putative double-stranded DNA (dsDNA)-binding fold (21). This complex (Fig. 1) not only targets BRCA2 to dsDNA and/or ssDNA junctions typical of replication-associated lesions, DSBs, or DSGs but also displaces RPA to allow loading of the recombination enzyme RAD51 (46).

RAD51 must first form a helical nucleoprotein filament on ssDNA. The RAD51-ssDNA filament subsequently mediates synapsis with homologous dsDNA in the sister chromatid to initiate the strand exchange required for repair by HR. Getting these reactions to proceed in the right order presents a challenge, because under physiological ionic conditions, human RAD51 preferentially forms stable complexes on dsDNA rather than ssDNA.

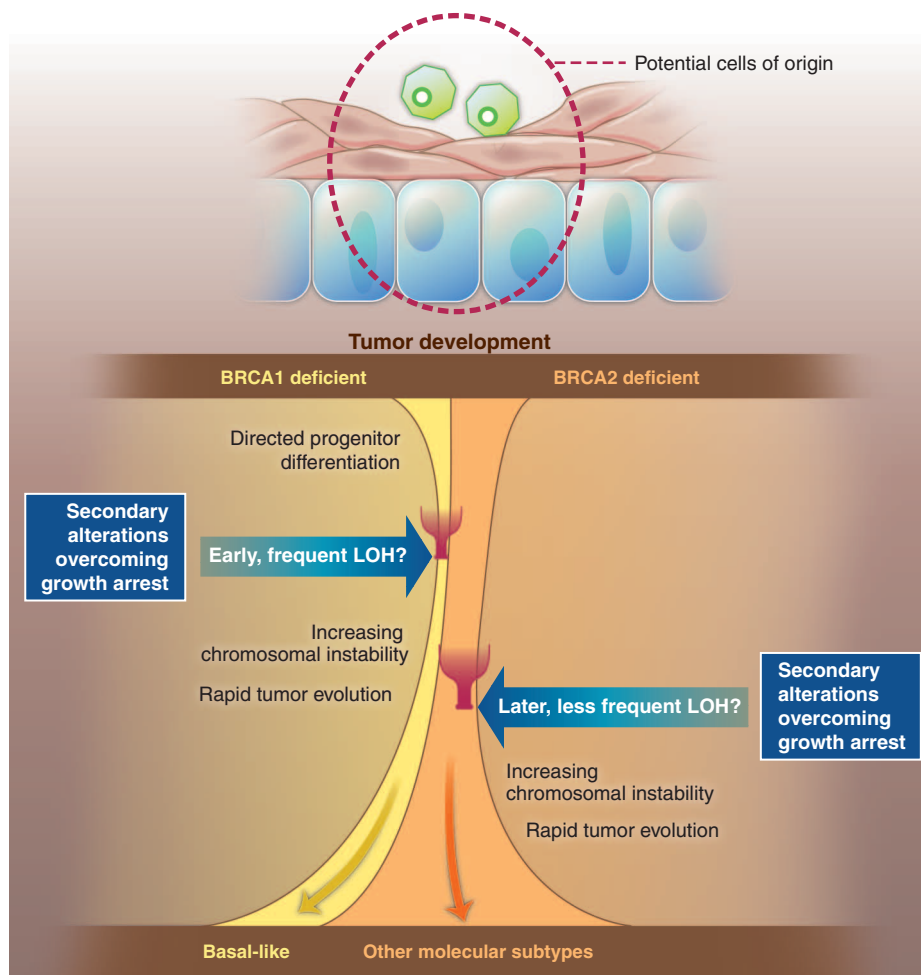


Fig. 2. The chromosomal instability model for the pathogenesis of BRCA-deficient breast cancer. The potential cell of origin depicted in the red dotted circle (blue, luminal epithelium; brown, myoepithelium; green, progenitor cell) is debated. BRCA1 deficiency forces differentiation toward basal-like characteristics, whereas BRCA2 deficiency does not. LOH provokes chromosomal aberrations, which lead to cell growth arrest (red bottleneck). Further genetic alterations, such as those inactivating cell cycle checkpoints, allow cells to bypass this bottleneck and resume growth. Chromosomal instability in the absence of functional BRCA proteins allows rapid evolution of tumors with basal-like (BRCA1-deficient) or different (BRCA2-deficient) characteristics. In contrast to BRCA1, there is evidence that BRCA2 LOH may be intratumorally heterogeneous and dispensable for carcinogenesis, which leads to the speculation that LOH occurs later in BRCA2-deficient tumor evolution. [Figure adapted from (65)]

BRCA2 binds directly to RAD51, and regulates RAD51's DNA substrate selectivity, to overcome this challenge. RAD51 binding is mediated by the BRC repeats in BRCA2 (8, 12). These are motifs of ~30 to 40 residues each (Fig. 1), present in eight copies in all known mammalian BRCA2 molecules, whose sequence and spacing is evolutionarily conserved (47, 48). Binding of the BRC repeats to RAD51 via an interface comprising two distinct modules (20, 49) stabilizes RAD51 filament formation on ssDNA while inhibiting RAD51-dsDNA binding (50, 51). These opposing activities bolster one another to correctly order the HR reaction, first targeting RAD51 to ssDNA for pre-synaptic filament formation before synapsis with the homologous duplex (Fig. 1). This RAD51-targeting activity is exhibited by single BRC repeat peptides, a segment of human BRCA2 containing all eight BRC repeats, or the full-length human BRCA2 protein (50–54), which exemplifies the segmental nature of BRCA2 structure-function relations and suggests that the biochemistry of these functional segments accurately reflects that of the full-length protein.

Observations made more than 10 years ago (55) show that stalled replication forks in BRCA2-deficient cells collapse into DSBs after genome-wide replication fork stalling induced by prolonged exposure to hydroxyurea (HU), an agent that depletes nucleotide pools required for DNA synthesis. This suggests that BRCA2 stabilizes the structure of arrested forks to allow their error-free resolution by HR. More recent observations extend these prior findings (56), by showing that a C-terminal segment of BRCA2—dispensable for chromosomal DSB repair by HR—protects nascent DNA strands at stalled forks, after brief HU exposure, from degradation by the MRE11 nuclease.

BRCA1 acquires E3 ubiquitin ligase enzymatic activity (57–59) by heterodimerization with BARD1 (60), through their N-terminal RING domains. In humans, BRCA1 mutations in the RING domain that disengage it from BARD1 and lead to the loss of E3 ligase activity are frequently associated with cancer predisposition. At first glance, these observations suggest that BRCA1 and BARD1 may exert their functions primarily through their conjoint E3 ligase activity. However, an engineered mutation Ile(or Val)26Ala in the BRCA1 RING domain that removes E3 ligase activity without perturbing the BARD1 interaction reveals that DNA damage responses triggered by certain agents (e.g., irradiation and topoisomerase inhibitors) are independent of E3 ligase activity (61, 62), as is breast cancer suppression in mouse strains that harbor such an engineered mutation (63). On the other hand, a cancer-associated BRCA1 RING mutation Cys61Gly that reduces both E3 ligase activity and BARD1 binding is essential for breast cancer suppression (64). These varied observations highlight gaps in our understanding of the relevance of BRCA1-BARD1 E3

ligase activity to chromosome integrity and tumor suppression.

Protection Against Aneuploidy During Chromosome Segregation

The numerical chromosomal aberrations (aneuploidy) found in BRCA-deficient cells likely emanate from several causes. Transgression of the G₂ checkpoint for DNA damage, anomalies in spindle formation and the mitotic checkpoint at the metaphase-anaphase transition, and defects in the completion of cell division by cytokinesis have all been reported after the inactivation of BRCA1 or BRCA2 in several different experimental models [reviewed in (65)].

Although BRCA1-deficient cells can complete mitosis, the fidelity of chromosome segregation is frequently compromised, which leads to the appearance of aneuploid progeny. The BRCA1-BARD1 heterodimer (66) regulates mitotic spindle assembly, localizing at the spindle poles where its E3 ligase activity recruits the essential spindle assembly factor TPX2. BRCA1-BARD1 has also been implicated in spindle microtubule organization via attenuation of HMMR, a putative motility receptor for hyaluronan, in which polymorphisms have been identified that correlate with an increased risk of breast cancer in an Israeli population (67). Phosphorylation of BRCA1 by the checkpoint kinase CHK2 may regulate these functions (68).

In BRCA2-deficient cells, aneuploid divisions likely arise from defects in the mitotic spindle assembly checkpoint (69), as well as the defective completion of cell division by cytokinesis at the stage of daughter cell abscission (70–72). Moreover, BRCA2, like BRCA1, has been implicated in controlling mitotic entry during recovery from the G₂ checkpoint for DNA damage (73, 74), which raises the possibility that DNA damage persisting into mitosis affects later events during chromosome segregation. At the spindle assembly checkpoint, BRCA2 recruits the PCAF acetyltransferase to the kinetochores of metaphase chromosomes (69), which enables the acetylation of the checkpoint protein BubR1. BRCA2 deficiency decreases BubR1 acetylation and the stability of BubR1, which leads to weakened checkpoint activity and aneuploidy. BRCA2 inactivation in zebrafish, murine, or human cells (70–72, 75, 76) increases the frequency of failure to complete cell division by cytokinesis, which engenders multinucleate structures with ≥4N DNA content. This defect occurs at a low frequency in murine cells heterozygous for a germline truncation in *Brca2* (70), as well as in human cells compound-heterozygous for *BRCA2* mutations found in Fanconi anemia patients (76). Filamin A recruits BRCA2 to the cytokinetic midbody, where it controls assembly of the ESCRT (endosomal sorting complex required for transport) protein complex implicated in cytokinetic abscission, a function impaired by a human cancer-associated *BRCA2* mutation (72).

Collectively, these observations suggest that BRCA1 and BRCA2 act in distinct mechanisms to enhance the fidelity of chromosome segregation, although they are not essential components of the mitotic machineries for spindle assembly, kinetochore-microtubule attachment, or cytokinesis. Segregation defects provoked by BRCA deficiency may not only generate the copy number variations and whole-chromosome aneuploidies thought to promote tumorigenesis (77) but also cooperate with the subtler, but frequent, structural variations triggered by HR defects (29). Thus, BRCA1 and BRCA2 may be particularly effective as tumor suppressors through their extended role as chromosome custodians across interphase as well as mitosis.

Emerging Functions

Additional functions have been proposed, particularly for BRCA1 but also for BRCA2. These include roles [reviewed in (60)] in chromatin remodeling and gene expression, in RNA polymerase II-associated complexes implicated in DNA damage responses, in the regulation of mRNA polyadenylation, in an E3 ligase complex that modifies p53, in protecting telomeres that cap chromosome ends, and in heterochromatin maintenance and retrotransposon silencing by the control of site-specific DNA methylation (78). Whether these functions are salient to tumor suppression by the BRCA proteins is not yet clear.

A recent report (79) proposes that decreased ubiquitylation of histone H2A in BRCA1-deficient cells alters the structure of constitutive heterochromatin that flanks the centromeres of chromosomes, activating transcription from normally silent genomic regions (called “satellite sequences”) therein. Ectopic expression of satellite transcripts reproduces several effects of BRCA1 deficiency, such as DNA breakage and abnormal chromosome segregation. However, an Ile26Ala BRCA1 mutant protein lacking E3 ligase activity that does not support H2A ubiquitylation or satellite repression *in vitro* (79) nevertheless effectively suppresses tumorigenesis *in vivo* (63).

The “Chromosomal Instability” Model for Carcinogenesis in BRCA Mutation Carriers **Genetic Progression and Clinical Features**

How well does a “chromosomal instability” model (Fig. 2) explain the clinical features of cancer susceptibility in individuals who carry *BRCA* gene mutations? As predicted by a model in which chromosomal instability provoked by BRCA inactivation accelerates the process of genetic variation and selection that drives carcinogenesis, *BRCA* mutation carriers develop breast and ovarian cancers at an earlier age than noncarriers. Moreover, primary cancers of the contralateral breast occur very frequently in mutation carriers (80), which suggests that ongoing chromosomal instability generates a “field” of cells susceptible to transformation. One puzzle in this regard is

that *BRCA* gene heterozygosity suffices for cancer predisposition, although it does not provoke the high levels of chromosomal instability typical of cells lacking both copies of either *BRCA1* or *BRCA2* (5, 6, 81). This raises the possibility that heterozygosity has a dosage effect sufficient to trigger low, but quantitatively significant, levels of genome instability that accumulates over many cell divisions. Alternatively, certain heterozygous mutations may act in a dominant manner [for example, (82)].

Biallelic inactivation of *BRCA1* or *BRCA2* not only triggers profound chromosomal instability but also quickly leads to cell cycle arrest or apoptosis because of the activation of checkpoint mechanisms that monitor the cell cycle (Fig. 2). Indeed, in murine models, concordant inactivation of checkpoint mechanisms mediated by *Tp53* is essential for tumorigenesis in this setting (83–86). Thus, an important implication of the chromosomal instability model is that homozygous loss of both copies of either *BRCA1* or *BRCA2* in premalignant cells must be preceded (or at least, quickly followed) by inactivation of checkpoint mechanisms that would otherwise eliminate the *BRCA*-deficient cells (Fig. 2). Consistent with this notion, several studies report an increased frequency of *p53* mutations in *BRCA*-deficient cancers [e.g., (87, 88)]. Not all *BRCA*-deficient tumors harbor *p53* mutations, however, which suggests that alternative routes to checkpoint inactivation in this setting probably exist. Indeed, *BRCA1* and *BRCA2* have themselves been implicated in checkpoint control.

Whereas germline inheritance of a single mutant allele of *BRCA1* or *BRCA2* predisposes to cancer, somatic deletion of the remaining wild-type allele (also termed “loss of heterozygosity” or LOH) is considered essential for carcinogenesis. However, emerging evidence suggests that germline heterozygous *BRCA2* mutations may suffice for carcinogenesis in several tissues, with LOH either absent or occurring relatively late in disease progression. Only 13 of 25 high-grade serous ovarian cancer samples from *BRCA2* mutation carriers exhibited LOH, defined as a >20% increase in mutant allele frequency compared to paired germline samples (89). In invasive prostate carcinomas from men carrying a *BRCA2* mutation, LOH was detected in only 5 of 10 samples (and none of the high-grade intraepithelial neoplasia from the same samples) (90). Heterogeneous intratumoral LOH was observed in invasive breast cancers from *BRCA* mutation carriers (91). Only one out of four invasive pancreatic ductal adenocarcinomas arising in *BRCA2*^{99delC} carriers (85) and three out of four in *BRCA2*^{6174delT} carriers (92) exhibited LOH. Although these small sample numbers warrant due caution, these analyses suggest that a varying, but perhaps substantial, fraction of cancers arising in *BRCA2* mutation carriers may retain an intact second allele, with the frequency of LOH increasing with advancing

disease. By comparison, *BRCA1* LOH was detected in 30 of 32 serous ovarian carcinomas, and 2 of 4 advanced pancreatic cancers, from patients carrying *BRCA1* germline mutations. Epigenetic silencing of *BRCA1* has also been reported.

Tissue Specificity

Observed differences in the frequency of LOH in cancers from *BRCA1* versus *BRCA2* mutation carriers draw attention to other distinguishing features. *BRCA1* mutation carriers primarily experience a marked excess of ovarian and female breast cancers, whereas inherited *BRCA2* mutations also cause significant predisposition to cancers of the male breast, pancreas, prostate, and other organs (93, 94). Moreover, *BRCA1*-deficient breast cancers predominantly exhibit characteristics of the “basal-like” subgroup (95), such as the lack of estrogen receptor (ER) expression, which is not evident in the majority of *BRCA2*-deficient tumors (Fig. 2). These clinical characteristics are consistent with laboratory experiments (96–99) suggesting that *BRCA1* mutations affect the early differentiation of breast tissue from ER-negative progenitors of debated lineage and so promote carcinogenesis through tissue-specific functions distinct from those responsible for chromosome integrity. Such tissue-specific functions of *BRCA1* may also help to explain the predominance of breast and ovarian cancer susceptibility in mutation carriers.

Why cancer predisposition associated with *BRCA* gene mutations should occur in specific tissues such as the breast, ovary, pancreas, or prostate has not yet been satisfactorily explained. Tissue-specific functions affecting gene expression or differentiation—such as those discussed above for *BRCA1*—have been proposed. Others may become apparent when *BRCA* disruption renders cells in particular tissues more sensitive to the effects of local mutagens or to periods of rapid cell division. An alternative hypothesis is that tumors can arise only in tissues that are permissive for the prolonged survival of cells lacking both *BRCA* alleles (65, 100), which allows time for secondary mutations affecting genes like *p53* that permit tumor progression and outgrowth. Prophylactic oophorectomy in women who carry *BRCA* mutations reduces the risk of breast cancer, which suggests that ovary-derived factors may foster the outgrowth of malignant epithelial cells (101, 102), particularly in *BRCA2* mutation carriers. However, recent work suggesting that a significant fraction of *BRCA2*-deficient tumors arising in the target tissues of germline mutation carriers retains an intact second allele diminishes the likelihood that this hypothesis will provide a satisfying general explanation for tissue specificity, at least in the case of *BRCA2*.

Rationalizing Approaches to Therapy

The role of the *BRCA* proteins as custodians of chromosome integrity during the cell cycle offers

the potential for new approaches to therapy. The inability of *BRCA*-deficient cells to deal correctly with stalled DNA replication forks underpins their sensitivity to classical cancer chemotherapies that arrest replication. These drugs include well-established DNA cross-linking agents, such as platinum compounds, which are showing clinical promise in *BRCA1*-deficient cancer patients (103, 104). In addition, new targeted agents have been developed that inhibit the enzyme poly (ADP)-ribose polymerase 1 (PARP1) and block the DNA repair pathway that removes damaged nitrogen bases by base excision repair to leave behind a ssDNA gap that effectively arrests replication forks. These drugs have shown excellent efficacy in laboratory studies (105, 106) and are being extensively tested in different clinical settings.

Clinical trials of highly potent PARP1 inhibitors (PARPi) have thus far produced a mix of encouraging (107–109) and disappointing (110, 111) results. Further optimization of treatment regimes and better selection of patients are required to maximize their potential. Three major issues warrant consideration. First, there is evidence that a fraction of tumors arising in mutation carriers retain a wild-type *BRCA2* allele [for example, (86, 90, 91)], which confers *de novo* primary resistance to PARPi. Patients with such tumors would be unlikely to benefit from the drugs. Second, chromosomal instability in *BRCA*-deficient cancer cells may enhance the risk of further mutations that induce secondary treatment resistance, including reversions that partially restore inactive *BRCA* gene function (112, 113) particularly in patients previously treated with platinum compounds (114). Finally, diminished (but not absent) HR in the nontumorous somatic tissues of heterozygous *BRCA* mutation carriers could potentiate the toxicity of regimes combining PARPi with systemic genotoxins like anthracyclines or nucleoside analogs. It could also potentiate iatrogenic carcinogenesis after long-term (potentially preventative) PARPi treatment. These problems are raised by the observation that DNA breakage marked by γ H2AX is transiently induced even in nontarget tissues in *BRCA* mutation carriers treated with PARPi (115). Treatment regimes that better limit exposure or new methods that target drug delivery to tumor tissues could help to address these problems.

Emerging evidence that essential HR proteins other than *BRCA1* and *BRCA2* may be inactivated by somatic mutations or epigenetic silencing in certain tumor types has begun to impact the treatment of non-*BRCA* mutant sporadic cancers [reviewed in (116)]. For instance, it has been proposed that certain sporadic ovarian cancers or triple-negative breast cancers devoid of *BRCA* mutations may nevertheless exhibit defects in HR. If correct, this premise could help to explain variations in clinical sensitivity to replication-arresting chemotherapies, such as

platinum compounds, or targeted agents like PARP1 inhibitors.

Conclusion

In summary, the past 20 years have witnessed remarkable advances in our understanding of the biological functions of BRCA1 and BRCA2 as custodians of chromosome integrity across the cell cycle that underlie their potent tumor-suppressor activity. In a broader context, this body of work has revealed causal relations between chromosomal instability and susceptibility to common forms of epithelial cancer, has provided a powerful experimental landscape in which to explore the biological mechanisms that preserve chromosome integrity, and has allowed the formulation of a conceptual framework to interpret the role of these mechanisms in carcinogenesis and to exploit them for cancer therapy. There is, however, clearly so much more to do that perhaps the best is yet to come.

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Supplementary Materials

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Fig. S1

Reference (117)

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Myeloid cells, innate lymphoid cells, and the cytokines they secrete cooperate to maintain immune tolerance in the gut.

Microbiota-Dependent Crosstalk Between Macrophages and ILC3 Promotes Intestinal Homeostasis

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Introduction: The gastrointestinal tract is colonized by an extraordinarily large number of commensal microbes and is constantly exposed to ingested antigens and potential pathogens. Regulation of intestinal tolerance thus represents the main task of the immune system of the gut mucosa. Accumulated evidence suggests that gut commensals contribute to the maintenance of intestinal homeostasis, partly through their ability to control the differentiation of effector T lymphocytes in the mucosa and to modulate inflammatory responses through the induction of regulatory T cells (T_{reg}) and interleukin-10 (IL-10) production. Tissue-resident mononuclear phagocytes (MNPs), including macrophages (MPs) and dendritic cells (DCs), are specifically equipped to detect a wide range of microbial signals and to capture, process, and present extracellular antigenic material to T lymphocytes. MNPs have been shown to contribute to the maintenance of intestinal immune tolerance through the induction or expansion of T_{reg} in the intestine. Despite their key role in microbial sensing and immune tolerance, the cellular and molecular cues that translate microbial signals into immunoregulatory MNPs in the intestine are not completely understood.

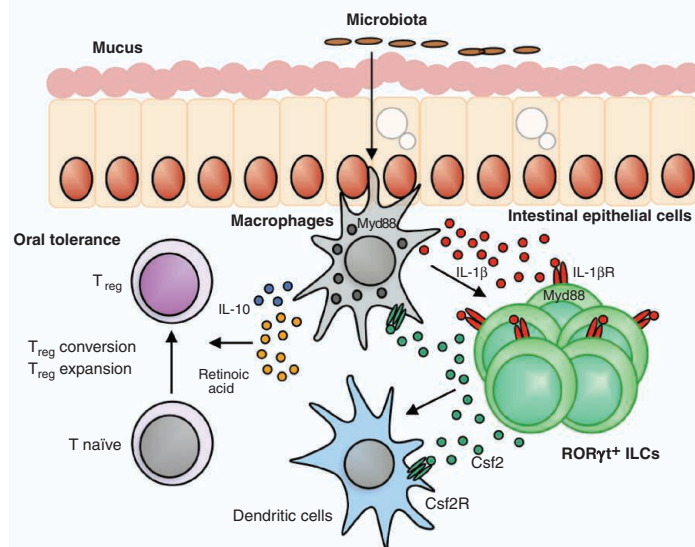
Rationale: The cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF), recently renamed Csf2, is a key determinant of myeloid lineage differentiation and is required for the optimal function of tissue MNPs. Recent results from our laboratory revealed that although Csf2-deficient mice have normal numbers of lymphoid tissue-resident DCs, they display significantly reduced numbers of steady-state nonlymphoid tissue-resident DCs in the small intestine, including the lamina propria CD103⁺CD11b⁺ DC subset implicated in the induction of lamina propria T_{reg} . These results prompted us to further explore the contribution of Csf2 to intestinal immune homeostasis in vivo. We used detailed profiling

and functional immune assays of the MNP and lymphocyte compartment in the gut, as well as genetically engineered mice that lack Csf2 or the transducer Myd88 specifically in MNPs or lymphocytes, to explore the role of MNPs in the maintenance of immune homeostasis in the gut.

Results: Our results revealed a crosstalk between IL-1 β -secreting MPs and Csf2-producing ROR γ t⁺ type 3 innate lymphoid cells (ILC3) in the intestinal mucosa. Microbiota-driven IL-1 β production by MPs promoted the release of Csf2 by ILC3, which in turn controlled DCs and MPs to maintain colonic T_{reg} homeostasis. Ablation of Csf2 reduced DC and MP numbers and impaired

their ability to produce regulatory factors such as retinoic acid (RA) and IL-10, leading to disrupted T_{reg} homeostasis in the large intestine. Conversely, administration of Csf2 cytokine increased T_{reg} frequency in the gut. Most notably, cell type-specific ablation of IL-1 receptor (IL-1R)-dependent signaling in ROR γ t⁺ ILC3 abrogated oral tolerance to dietary antigens and compromised intestinal immune homeostasis in vivo. Although the reduction in T_{reg} numbers was mostly observed in the large intestine, adoptive transfer studies in Csf2^{-/-} mice revealed impaired T_{reg} differentiation both in the small and large intestine, suggesting that Csf2-dependent MNP immunoregulatory functions control T_{reg} induction in both tissues.

Conclusion: This study established the commensal-driven MNP-ILC3-Csf2 axis as a key regulator of intestinal T cell homeostasis in the mouse intestine. Disturbance of this axis radically altered MNP



ILC3 translate microbial cues into immunoregulatory signals in the intestine. Microbial cues sensed by intestinal MPs lead to IL-1 β release. IL-1 β engages IL-1 receptor on ILC3, promoting Csf2 release. ILC3-derived Csf2 triggers DC and MP production of regulatory molecules (i.e., retinoic acid and IL-10), which in turn promotes the induction and expansion of T_{reg} . Csf2-primed DCs and MPs promote T_{reg} homeostasis locally and in mesenteric lymph nodes.

effector function, resulting in impaired oral tolerance to dietary antigens. These results represent an important advance in our understanding of how commensal microbes can regulate host intestinal immunity and may inform the design of novel immunotherapies for patients with inflammatory intestinal diseases with impaired GM-CSF function.

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Microbiota-Dependent Crosstalk Between Macrophages and ILC3 Promotes Intestinal Homeostasis

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The intestinal microbiota and tissue-resident myeloid cells promote immune responses that maintain intestinal homeostasis in the host. However, the cellular cues that translate microbial signals into intestinal homeostasis remain unclear. Here, we show that deficient granulocyte-macrophage colony-stimulating factor (GM-CSF) production altered mononuclear phagocyte effector functions and led to reduced regulatory T cell (T_{reg}) numbers and impaired oral tolerance. We observed that $ROR\gamma^t$ innate lymphoid cells (ILCs) are the primary source of GM-CSF in the gut and that ILC-driven GM-CSF production was dependent on the ability of macrophages to sense microbial signals and produce interleukin-1 β . Our findings reveal that commensal microbes promote a crosstalk between innate myeloid and lymphoid cells that leads to immune homeostasis in the intestine.

The gastrointestinal tract is colonized by an extraordinarily large number of commensal microbes and is constantly exposed to ingested antigens and potential pathogens. Regulation of intestinal tolerance thus represents the main task of the immune system of the gut mucosa. Defective immune tolerance in the gut is associated with the onset of inflammatory bowel diseases (IBD), a severe intestinal pathology that results from a dysregulated immune response to commensal microbes leading to chronic intestinal inflammation (1, 2). Accumulated evidence suggests that gut commensals contribute to the maintenance of intestinal homeostasis, partly through their ability to control the differentiation of effector T lymphocytes in the mucosa (3, 4) and to modulate inflammatory responses through the induction of T_{reg} s and interleukin-10 (IL-10) production (4–6).

Tissue-resident mononuclear phagocytes (MNP)s are equipped to detect a wide range of microbial signals and to capture and process extracellular antigens, including commensal microbial antigens in the form of peptide–major histocompatibility complexes (MHCs) that can be recognized by T lymphocytes (7). Mucosal tissue-resident MNP)s consist of two main cell populations, macrophages (MP)s and dendritic cells (DC)s (8). Tissue-resident macrophages are characterized as

MHCII⁺CD11c⁺CD103⁺CD11b⁺CX3CR1⁺F4/80⁺CD64⁺ cells, whereas tissue-resident DCs are characterized as MHCII⁺CD11c⁺CX3CR1^{int/–}F4/80[–]CD64[–] (fig. S1). DCs can further be subdivided into CD103⁺CD11b[–] (CD103⁺ DCs), CD103⁺CD11b⁺ (double-positive or DP DCs), CD103[–]CD11b⁺ (CD11b⁺ DCs), and CD103[–]CD11b⁺CD64⁺F4/80⁺ (MP) subsets (9–12) (fig. S1). Both DCs and macrophages have been shown to contribute to the maintenance of intestinal immune tolerance through the induction or expansion of T_{reg} s in the intestine (13–19). Despite their key role in microbial sensing and immune tolerance, the cellular and molecular cues that translate microbial signals into immunoregulatory MNP)s in the intestine remain poorly understood.

The cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF), recently renamed colony-stimulating factor 2 (Csf2), is a key determinant of myeloid lineage differentiation and is required for the optimal function of tissue MNP)s, including macrophages and DCs, thereby promoting host protection against environmental pathogens and vaccine responses (20, 21). Despite the key role of Csf2 in promoting MNP) survival, differentiation, and function, previous studies reported that mice lacking Csf2 or its receptor displayed only minor impairment in the development of spleen and lymph node DCs (22). Subsequent studies showing that Csf2 expression is increased in inflamed mice and that adoptively transferred monocytes generate DCs in the inflamed spleen but not in the steady-state spleen suggested that Csf2 is a major proinflammatory cytokine that controls the differentiation of inflammatory but not steady-state DCs in vivo (23, 24). These results are consistent with the contribution of Csf2 to the pathophysiology of numerous inflammatory and autoimmune diseases (25–27).

In contrast, we recently observed that although Csf2-deficient mice have normal numbers of lymphoid tissue-resident DCs, they display a significant

reduction in steady-state nonlymphoid tissue-resident DCs, including the CD103⁺CD11b⁺ DC subset found in the small intestine lamina propria (11, 28), which have been implicated in the induction of lamina propria T_{reg} s (14, 15). These results prompted us to further explore the contribution of Csf2 to intestinal immune homeostasis in vivo.

Regulation of Gut DC, Macrophage, and T_{reg} Cell Homeostasis by Csf2

We characterized the mucosal T cell compartment in Csf2-deficient mice ($Csf2^{-/-}$) in the steady state. Surprisingly, we observed a significant reduction in the frequency, number, and proliferation of CD45⁺TCR β ⁺CD4⁺Foxp3⁺ T_{reg} s in the colon of $Csf2^{-/-}$ mice compared to littermate controls (Fig. 1A and fig. S2A). The reduced T_{reg} number was specific to the colon and was not observed in the small intestine of $Csf2^{-/-}$ mice. The reduction in the number of colonic T_{reg} s was associated with a significant reduction in the frequency and number of IL-10[–] and IL-2–producing T cells, along with a significant increase in the number of colonic interferon- γ (IFN- γ)–producing T cells, whereas IL-17–producing T cells were unaffected in 6-week-old $Csf2^{-/-}$ mice compared to wild-type mice (Fig. 1B and fig. S2B). Histological analysis of colonic sections from Csf2-deficient animals did not reveal overt inflammatory infiltrates in the lamina propria (fig. S2C).

Because Csf2 plays a critical role in the differentiation and function of tissue MNP)s, we hypothesized that the alterations in T helper cell subsets observed in the colon of $Csf2^{-/-}$ animals might be due to defects in mucosal MNP)s. Accordingly, we found reduced numbers of colonic DCs and macrophages in the absence of Csf2 (Fig. 1, C and D), thus establishing an important role for Csf2 in the homeostasis of the colonic MNP) pool. DCs and macrophages have been reported to generate Foxp3⁺ T_{reg} s, via the production of the regulatory mediators retinoic acid (RA) and IL-10 in the presence of transforming growth factor- β (TGF- β). Thus, we analyzed the capacity of DCs and macrophages to produce these regulatory mediators in the absence of Csf2. We observed a significant reduction in the activity of the RA-generating enzyme retinaldehyde dehydrogenase (ALDH) throughout all colonic DC subsets and macrophages in $Csf2^{-/-}$ mice (Fig. 1E and fig. S2D) associated with reduced expression of *Aldh1* transcripts (fig. S2, E and F). Absence of Csf2 was also associated with a significant reduction in the release of TGF- β by colonic CD103⁺CD11b⁺ DCs and with reduced IL-10 secretion by macrophages (Fig. 1, F and G), which extends previous observations showing that Csf2 controls IL-10 and TGF- β release by peritoneal macrophages upon uptake of apoptotic cells (29). Notably, expression of *Aldh1a2* and *Il10*, and release of IL-10 were restored in $Csf2^{-/-}$ macrophages upon addition of exogenous Csf2 (fig. S2, G and H).

These findings suggest that the absence of Csf2 results in a reduction in the number, frequency,

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and function of DCs and macrophages in the colon. We thus sought to determine whether the alterations in T helper cell subsets observed in *Csf2*^{-/-} animals was due to impaired MNP function. Accordingly, we found that colonic macrophages and DCs isolated from *Csf2*^{-/-} mice were compromised in their ability to drive T_{reg} differentiation ex vivo compared to their *Csf2*^{+/+} counterparts

(Fig. 1H). This was reversed upon addition of exogenous Csf2 (Fig. 1H), suggesting that the reduced T_{reg} pool observed in *Csf2*^{-/-} mice was a consequence of impaired mucosal MNP function and not only reduced MNP numbers. Critically, administration of B16 melanoma cells that overexpressed Csf2 to *Csf2*^{-/-} mice restored MNP numbers (fig. S3A) and increased T_{reg} frequency (Fig. 1I), while

reducing the number of IFN γ -producing intestinal T cells to levels comparable to those of untreated C57Bl/6 mice (fig. S3B), consistent with the ability of exogenous Csf2 to restore the immunoregulatory potential of *Csf2*^{-/-} macrophages and DCs ex vivo (Fig. 1H). Together, these data establish Csf2 as a master regulator of MNP immunoregulatory function in the steady-state colon tissue.

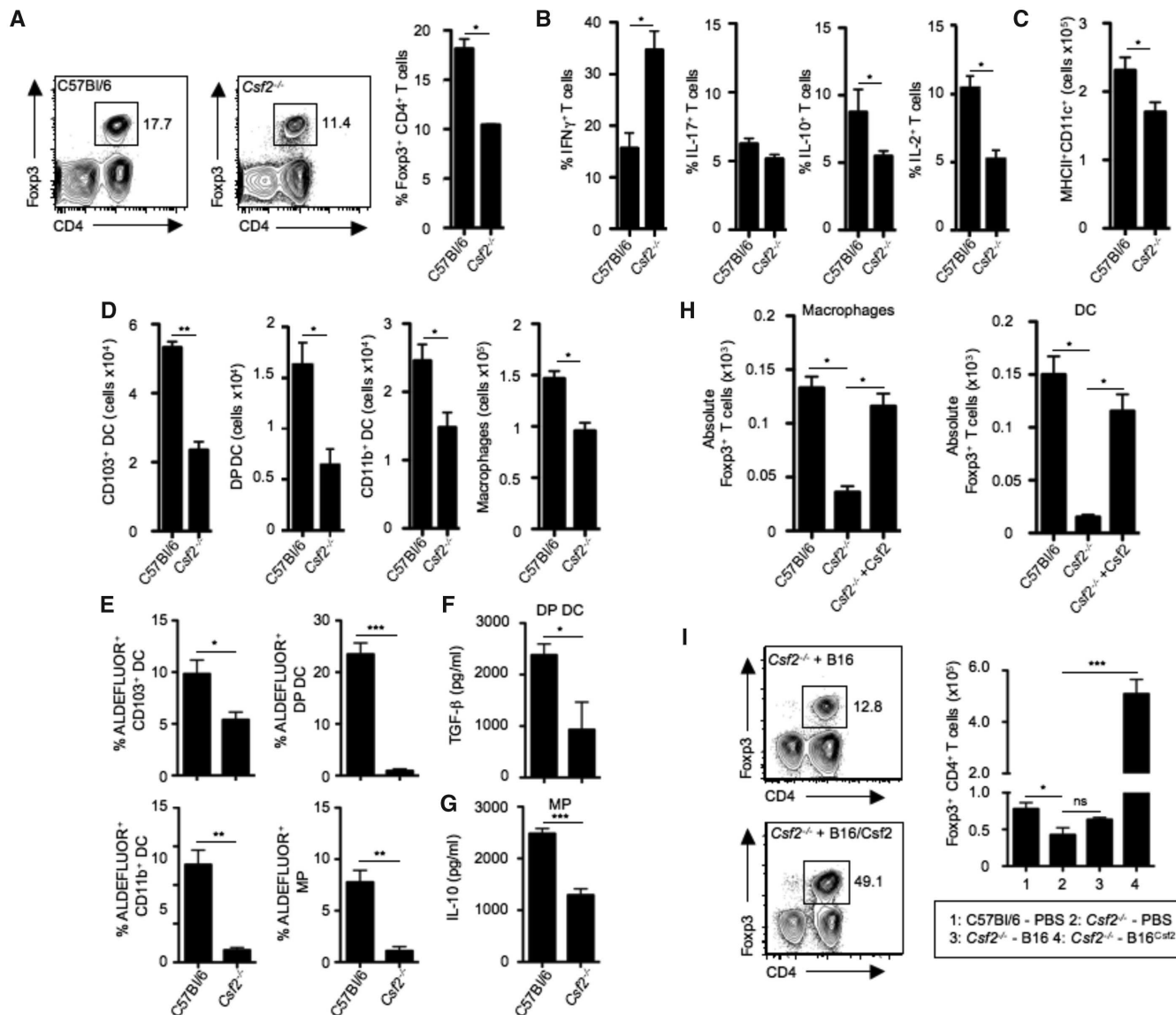


Fig. 1. Csf2 regulates tissue mononuclear phagocyte frequency and effector functions required for T helper cell homeostasis. (A) Contour plots and bar graph show percentages of CD3⁺CD4⁺Foxp3⁺ colonic T_{reg}s among total colonic lamina propria CD45⁺ cells in C57Bl/6 and *Csf2*^{-/-} mice. (B) Bar graphs show percentages of CD3⁺CD4⁺ T cells producing IFN γ , IL-17, IL-10, or IL-2 among total colonic CD45⁺ cells in C57Bl/6 and *Csf2*^{-/-} mice after 4 hours of stimulation with phorbol 12-myristate 13-acetate (PMA)–ionomycin in the presence of Brefeldin A. (C) Bar graphs show absolute numbers of MHCII⁺CD11c⁺ cells among total colonic CD45⁺ cells in C57Bl/6 and *Csf2*^{-/-} mice. (D) DCs were characterized as CD45⁺MHCII⁺CD11c⁺ and further subdivided into CD103⁺ DCs, CD103⁺CD11b⁺ DCs [double positive (DP) DCs], and CD11b⁺ DCs. Macrophages (MP) were characterized as MHCII⁺CD11c⁺CD11b⁺F4/80⁺CD64⁺ cells. Bar graphs show absolute numbers of CD103⁺ DCs, DP DCs, CD11b⁺ DCs, and MPs in C57Bl/6 and *Csf2*^{-/-} mice. (E) Percentages of ALDEFLUOR⁺ cells among each colonic DC

subset and MPs in C57Bl/6 and *Csf2*^{-/-} mice. (F and G) Bar graphs show enzyme-linked immunosorbent assay (ELISA) measurement of TGF- β production by sorted DP DCs (F) and IL-10 production by sorted MPs (G). (H) Colonic MPs or DCs were FACS-sorted from C57Bl/6 or *Csf2*^{-/-} mice and cocultured with naïve T cells alone or in the presence of exogenous Csf2. Bar graphs show absolute numbers of Foxp3⁺ T_{reg}s. Data are representative of six independent experiments and are shown as mean $\pm$ SD. (I) Contour plots show percentages of CD45⁺CD3⁺CD4⁺Foxp3⁺ colonic T_{reg}s in *Csf2*^{-/-} mice 12 to 14 days after injection with B16 melanoma cells or B16 cells overexpressing Csf2 (B16^{Csf2}). Bar graphs show absolute number of colonic T_{reg}s in the indicated groups. All data (A to H) are shown as mean $\pm$ SD of three independent experiments with at least three mice per experiment. Student's *t* test (A to F) and one-way analysis of variance (ANOVA) Bonferroni's multiple comparison test (H and I) were performed. Statistical significance is indicated by **P* < 0.05, ***P* < 0.01, ****P* < 0.001; ns, not significant.

Notably, blockade of RA production [with 4-diethyl-aminobenzaldehyde (DEAB)] and/or blockade of IL-10 [with monoclonal antibody (mAb) against IL-10] abrogated the ability of Csf2 to rescue T_{reg} induction in vitro by $Csf2^{-/-}$ MNPs, whereas addition of RA rescued T_{reg} induction in these cultures (fig. S3, C and D). Consistent with these findings, injection of the RA receptor antagonist LE540 compromised Csf2-mediated rescue of T_{reg} in $Csf2^{-/-}$ mice in vivo (fig. S3E), and conversely, injection of RA but not IL-10 restored T_{reg} frequency in $Csf2^{-/-}$ mice in vivo (fig. S3F).

Csf2 Is Produced by ROR γ ⁺ ILC3

Our results showing that colonic T_{reg} homeostasis was dependent on Csf2 prompted us to characterize the source of Csf2 in a noninflamed intestine. Previous reports have suggested that Csf2 is primarily produced by radio-resistant epithelial cells (30), including Paneth cells (31) in the gut. Surprisingly, we found that in the large and small

intestine, Csf2 was constitutively produced by tissue-resident CD45⁺ hematopoietic cells expressing the retinoic acid-related orphan receptor γ t (ROR γ t) (Fig. 2A). ROR γ ⁺ T helper 17 (T_H 17) cells can produce large amounts of Csf2 in the inflamed brain and intestine (26, 27, 32). Other ROR γ -dependent cell populations include group 3 innate lymphoid cells (ILC3) composed of lymphoid tissue-inducer (LTi) cells and ROR γ ⁺ ILC expressing the natural killer (NK) cell receptor, NKp46, recently termed NCR⁺ILC3 cells (33).

LTi cells and NCR⁺ILC3 are highly abundant in the small and large intestine in the steady state (34). To test whether ROR γ ⁺ ILC3 contribute to the steady-state production of Csf2 in the intestine, we measured Csf2 production in $Rorc^{-/-}$ mice, which lack ROR γ -expressing cells (35). Csf2 expression was reduced in the large and small intestine of $Rorc^{-/-}$ mice and declined to levels almost as low as those found in $Csf2^{-/-}$ animals (Fig. 2B), suggesting that steady-state

production of the Csf2 cytokine in the intestine was predominantly mediated by ROR γ ⁺ cells. Accordingly, we observed that 80% of Csf2-producing cells in the normal small and large intestine resided within the CD3⁺CD45⁺ROR γ ⁺ compartment, consistent with the phenotype of ROR γ ⁺ ILC3 (Fig. 2, A and C). Csf2 was produced by both subsets of ROR γ ⁺ ILC3, including LTi cells and NCR⁺ ILC3, in the small and large intestine but not by NKp46⁺ROR γ ⁺ NK cells (33) (Fig. 2D). These results are consistent with previous data showing that human ROR γ -expressing NKp44⁺ ILC produce Csf2 (36). ROR γ ⁺ ILC3 are reportedly localized within the isolated lymphoid follicles (ILFs) (37, 38), so we used $Rorc^{+/EGFP}$ reporter animals to confirm that Csf2⁺ cells are enriched in ILF throughout the lamina propria (Fig. 2E and fig. S4, A to E). Areas enriched in ROR γ ⁺ cells (ILFs) also contained significantly higher levels of Csf2 transcripts when compared with intestinal epithelial

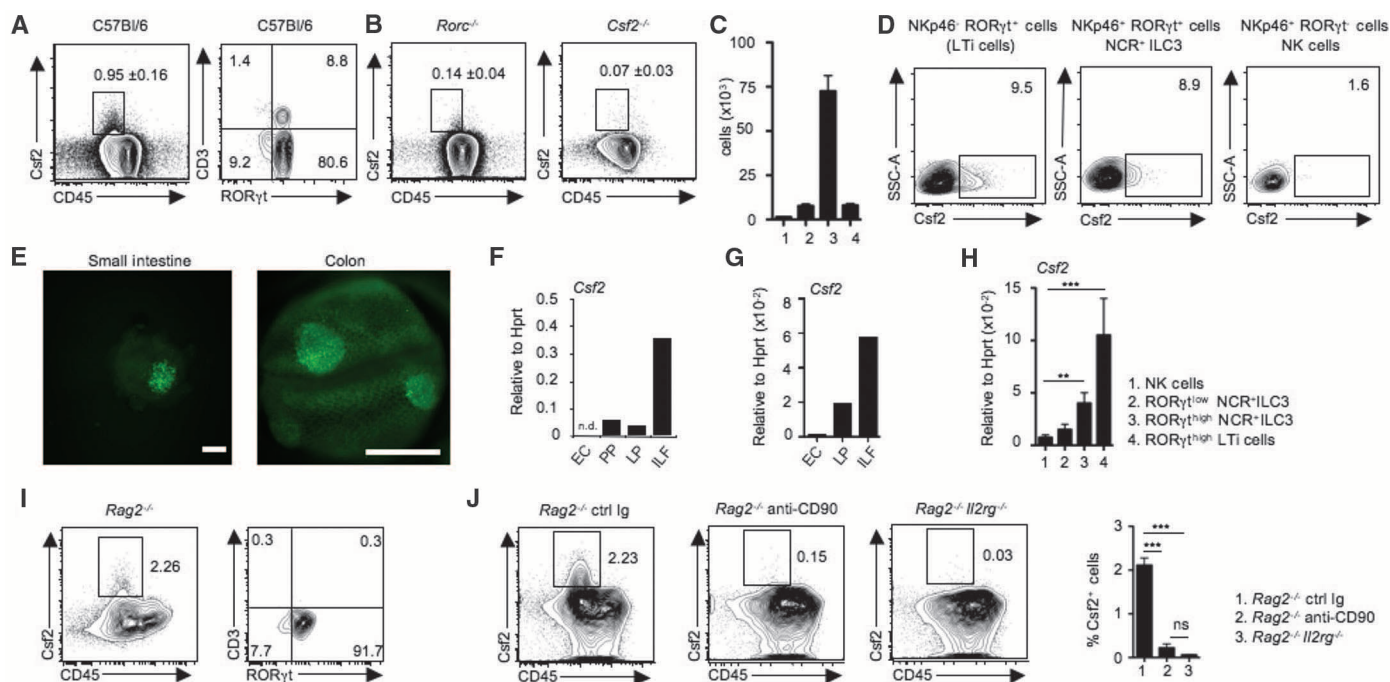


Fig. 2. ROR γ ⁺ ILCs are the major source of Csf2 in the steady-state gut. (A) FACS plots show Csf2 and CD45 expression in lamina propria cells and expression of CD3 and ROR γ t among gated Csf2⁺CD45⁺ lamina propria cells (data shown are representative of 10 independent experiments including at least 5 mice per experiment). Staining was performed on ex vivo isolated cells cultured for 4 hours in the presence of Brefeldin A. (B) FACS plots show percentage Csf2⁺CD45⁺ lamina propria cells in the small intestine of $Rorc^{-/-}$ and $Csf2^{-/-}$ mice. Data are representative of at least three experiments with two mice per group and are shown as mean $\pm$ SD. (C) Bar graph shows absolute numbers of Csf2-producing cells in lamina propria cells. 1: Csf2⁺CD3⁺ROR γ ⁺ T cells; 2: Csf2⁺CD3⁺ROR γ ⁺ T cells; 3: Csf2⁺CD3⁺ROR γ ⁺ ILCs; 4: Csf2⁺CD3⁺ROR γ ⁺ cells. (D) FACS plots show Csf2 expression on gated lamina propria NKp46⁺ROR γ ⁺ LTi cells, NKp46⁺ROR γ ⁺ NCR⁺ ILC3, and NKp46⁺ROR γ ⁺ NK cells. Data are representative of six independent experiments with at least three mice per group. (E) Representative fluorescence stereomicroscopic photographs of live lamina propria biopsy punches obtained from $Rorc^{+/EGFP}$ mice. Image shows clustered enhanced green fluorescent protein (EGFP) (ROR γ t) expression of ILF-residing $Rorc^{+/EGFP}$ cells isolated from small intestine (left; scale bar: 100 μ m) and colon (right; scale bar:

500 μ m). (F and G) Quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of Csf2 expression in isolated intestinal epithelial cells (EC), Peyer's patches (PP), lamina propria depleted of ILFs (LP), and ILF from small intestine (F) or colon (G). Data are representative of at least two independent experiments with three mice per group. (H) Quantitative RT-PCR analysis of Csf2 expression in colonic NK cells (1), ROR γ ⁺ NCR⁺ ILC3 (2), ROR γ ⁺ NCR⁺ ILC3 (3), and ROR γ ⁺ LTi cells (4) isolated from $Rorc^{+/EGFP}$ mice. (I) FACS plots show Csf2 and CD45 staining on total colonic lamina propria cells and expression of CD3 and ROR γ t among Csf2⁺CD45⁺ cells isolated from $Rag2^{-/-}$ mice (data are representative of two independent experiments with three mice per group). (J) FACS plots show Csf2 and CD45 staining on total colonic lamina propria cells isolated from either $Rag2^{-/-}$ mice, $Rag2^{-/-}$ mice injected with depleting anti-CD90 mAb, or $Rag2^{-/-}$ $Il2rg^{-/-}$ mice. Bar graph shows percentages of Csf2⁺ CD45⁺ cells in each group of mice. All Csf2 staining was performed on ex vivo isolated cells cultured for 4 hours in the presence of Brefeldin A. Data are shown as mean $\pm$ SD of two independent experiments with three mice per group. One-way ANOVA Bonferroni's multiple comparison test (H and J) was performed. Statistical significance is indicated by * P < 0.05, ** P < 0.01, and *** P < 0.001.

cells, Peyer's patches, and lamina propria depleted of ILF (Fig. 2, F and G, and fig. S4, A to E). These data identify ROR γ ⁺ ILC3 in ILFs as the main producers of intestinal Csf2 in the steady state.

Analysis of bone marrow chimeric mice that were lethally irradiated and then reconstituted with congenic hematopoietic progenitors revealed that host-derived ROR γ ⁺ ILC3 remained resident in the recipient intestine for several months after lethal body irradiation, consistent with previously published data (39). We observed high levels of Csf2 production in host-derived ROR γ ⁺ ILC3 even 3 months after lethal body irradiation (fig.

S5), suggesting that ROR γ ⁺ ILC3 likely contribute to the steady-state radio-resistant source of Csf2 reported by other investigators (30). Fluorescence-activated cell sorting (FACS) purification of ILC subsets from *Rorc*^{+/-EGFP} reporter animals further confirmed ROR γ ⁺ ILC3 as major producers of Csf2 (Fig. 2H). Accordingly, although Csf2-producing cells were detectable in high numbers in the small and large intestine of *Rag2*^{-/-} mice, which lack ROR γ -expressing T lymphocytes but not ILCs (Fig. 2I), they were reduced in ILC-deficient *Rag2*^{-/-}*Il2rg*^{-/-} mice and in *Rag2*^{-/-} mice depleted of ILCs with mAb

against CD90 (34) (Fig. 2J). ILC depletion in *Rag2*^{-/-} mice led to impaired RA-generating enzyme activity in colonic DCs and macrophages (fig. S6). Together, these results establish ILCs as a key producer of the myeloid regulatory cytokine Csf2 in the intestine.

Csf2 Production Is Dependent on Microbial Signals

The effector functions of ROR γ ⁺ ILC3, and the development and maturation of ILFs depend on commensal-driven signals (37, 40, 41). We found that Csf2 production was absent in newborns,

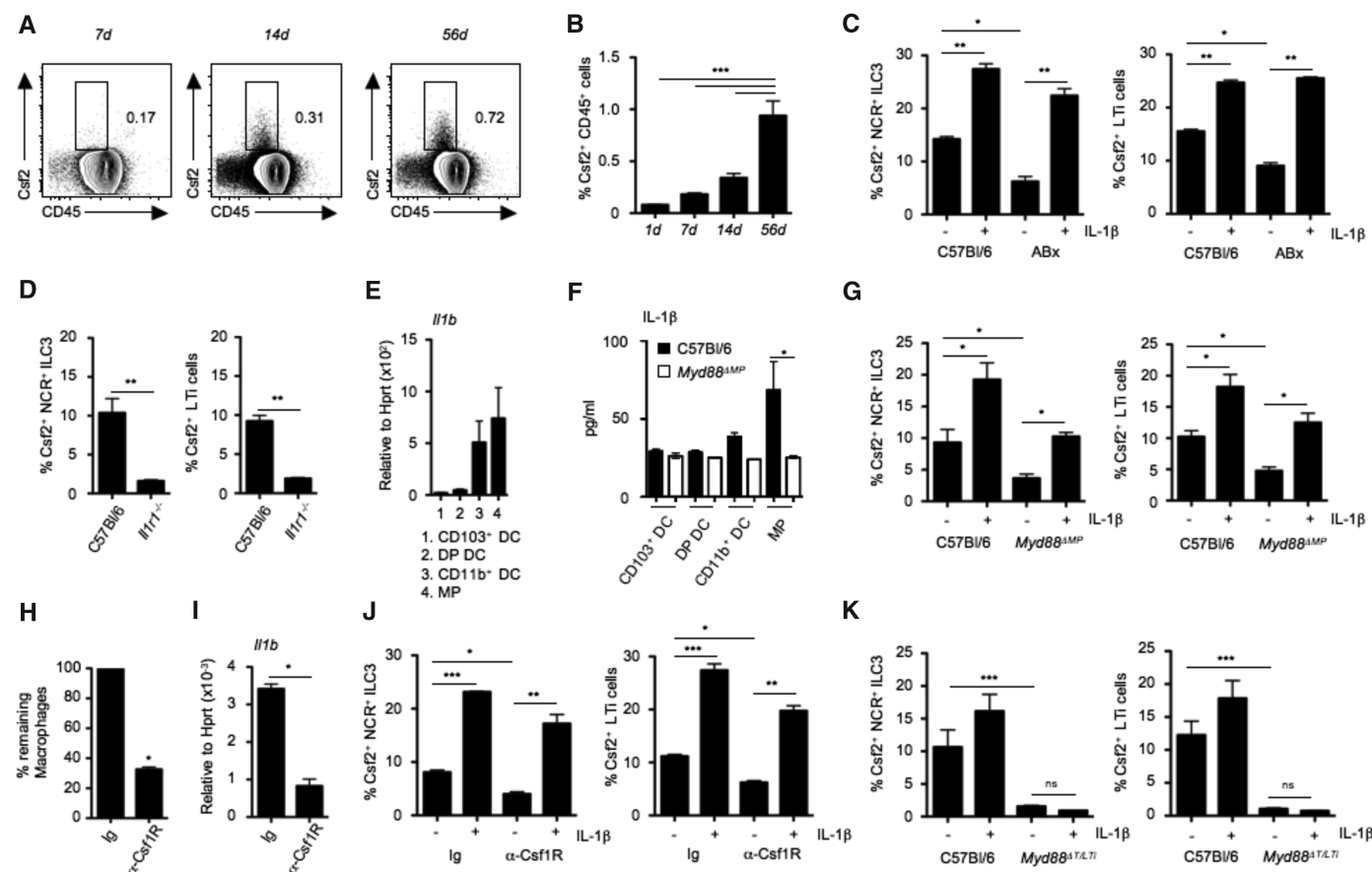


Fig. 3. Microbiota-driven IL-1 β release by intestinal macrophages regulates Csf2 production by ROR γ ⁺ ILC3. (A) FACS plot showing Csf2 expression in whole intestinal lamina propria CD45⁺ cells at the indicated time points after birth. (B) Bar graph shows percentages of Csf2⁺ cells among total lamina propria cells in mice at the indicated time points after birth. Data shown are the results of three independent experiments with at least three mice per group. (C) Percentages of Csf2⁺ cells among gated colonic lamina propria NCR⁺ ILC3 and LTI cells in conventional mice or mice treated with broad-spectrum antibiotics (ABx). Stains were performed on cells cultured for 4 hours with (+) or without (−) IL-1 β in the presence of Brefeldin A. Data are shown as mean $\pm$ SD of three independent experiments with at least three mice per group. (D) Percentages of Csf2⁺ cells among gated colonic lamina propria NCR⁺ ILC3 and LTI cells in *Il1r1*^{-/-} or C57Bl/6 mice. Data show the results of two independent experiments with three mice per group. (E) *Il1b* mRNA expression in FACS-purified colonic MNPs. Data shown are representative of two independent experiments with pooled cells of three mice. (F) IL-1 β protein production by purified intestinal DC subsets and MPs isolated from the colonic lamina propria of C57Bl/6 and *Myd88* ^{Δ MP} mice, measured by ELISA after 24 hours of culture in complete medium. Data are representative of two independent experiments with pooled cells of three mice. (G) Percent-

ages of Csf2⁺ cells among gated colonic ILC3 in *Myd88* ^{Δ MP} mice. Stains were performed in cells cultured for 4 hours with or without IL-1 β in the presence of Brefeldin A. Data are shown as mean $\pm$ SD of three independent experiments with at least three mice per group. (H) Groups of mice were injected with one injection of anti-Csf1R mAb (3 mg per mouse) or control mAb. Bar graph shows percentages of remaining macrophages in colonic tissue. (I) *Il1b* expression in whole colonic tissue of mice treated with anti-Csf1R mAb or isotype control. Data are shown as mean $\pm$ SD of at least three independent experiments with three mice per group. (J) Percentages of Csf2⁺ cells among gated colonic lamina propria NCR⁺ ILC3 and LTI cells in mice treated with anti-Csf1R mAb or control mAb. Staining was performed on total cells cultured for 4 hours with or without IL-1 β in the presence of Brefeldin A. Data are shown as mean $\pm$ SD of three independent experiments with three mice per group. (K) Csf2 production by colonic lamina propria NCR⁺ ILC3 and LTI cells in *Myd88* ^{Δ LTI} mice measured after 4 hours of culture with or without IL-1 β in the presence of Brefeldin A. Data are shown as mean $\pm$ SD of three independent experiments with at least three mice per group. Student's *t* test (D, H, and I) or one-way ANOVA Bonferroni's multiple comparison test (B, C, F, G, J, and K) were performed. Statistical significance is indicated by **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

slightly increased in 7-day-old mice, and increased substantially from day 14 after birth, concurrent with the increase in numbers and complexity of the intestinal microbial flora at these developmental stages (Fig. 3, A and B). To further investigate the influence of the commensal flora on Csf2 production in the intestine, we treated adult mice with broad-spectrum antibiotics known to strongly reduce the gut microbiota. In accordance with our findings in newborn animals, adult mice treated with broad-spectrum antibiotics displayed reduced Csf2 production in the small and large intestine (Fig. 3C). These results suggest that commensal-driven signals control the steady-state production of Csf2 by ROR γ ⁺ ILC3 in the mouse intestine.

Murine ROR γ ⁺ ILC3 lack Toll-like receptors (TLRs) and cannot directly sense microbial signals in the gut; hence, these cells must rely on other cellular sensors to translate cues from commensal bacteria into effector functions (42). We therefore explored whether cytokines derived from myeloid cells could drive Csf2 production by ROR γ ⁺ ILC3 ex vivo. Among several cytokines

tested, we observed that IL-1 β was a particularly potent inducer of Csf2 production by ROR γ ⁺ ILC3 (Fig. 3C), consistent with the reported role of IL-1 β as a potent driver of ILC function (38). Because the myeloid cytokine IL-23 promotes the production of the cytokine IL-22 by ROR γ ⁺ ILC3, we examined whether IL-23 also promoted Csf2 production by these cells (43). IL-23 was unable to promote Csf2 production by ROR γ ⁺ ILC3 (fig. S7A), whereas it stimulated the release of IL-22 (fig. S7B), as previously reported (43). Furthermore, IL-22 production by ROR γ ⁺ ILC3 was unaffected in *Csf2*^{-/-} mice (fig. S7C). Exposure to IL-1 β rescued Csf2 production by ROR γ ⁺ ILC3 isolated from antibiotic-treated mice (Fig. 3C). Accordingly, LT α and NCR⁺ ILC3 isolated from mice lacking IL-1 receptor 1 (*Il1r1*^{-/-}) failed to produce Csf2 (Fig. 3D), thereby implicating IL-1 β and IL-1R signaling as key drivers of Csf2 production in the intestine. In contrast, absence of the other IL-1 superfamily member, IL-18, did not compromise intestinal Csf2 production (fig. S7D). Together, these data indicate that IL-1 β —

producing cells that respond to microbial signals control the steady-state production of Csf2 by ROR γ ⁺ ILC3 in the intestine.

Sensing the commensal microflora by the TLR and the activation of the adapter protein Myd88 is critical for maintaining intestinal homeostasis (44) and leads to steady-state IL-1 β production by tissue MNP (45). Tissue-resident macrophages, CD103⁺ DCs, and CD103⁻ DCs arise from different developmental pathways and express distinct pattern recognition receptors (46). We found that intestinal macrophages were the highest producers of *Il1b* and IL-1 β protein, as previously reported (45, 47), suggesting that this population is a key regulator of Csf2 production in the gut (Fig. 3, E and F).

Because microbial signals were required to drive Csf2 production in the intestine, we next examined whether deletion of the TLR-adapter protein Myd88 in phagocytes influenced Csf2 production by ROR γ ⁺ ILC3. Lysozyme M (LysM) is expressed at high levels in macrophages relative to DCs (48), and notably, mice that lack Myd88

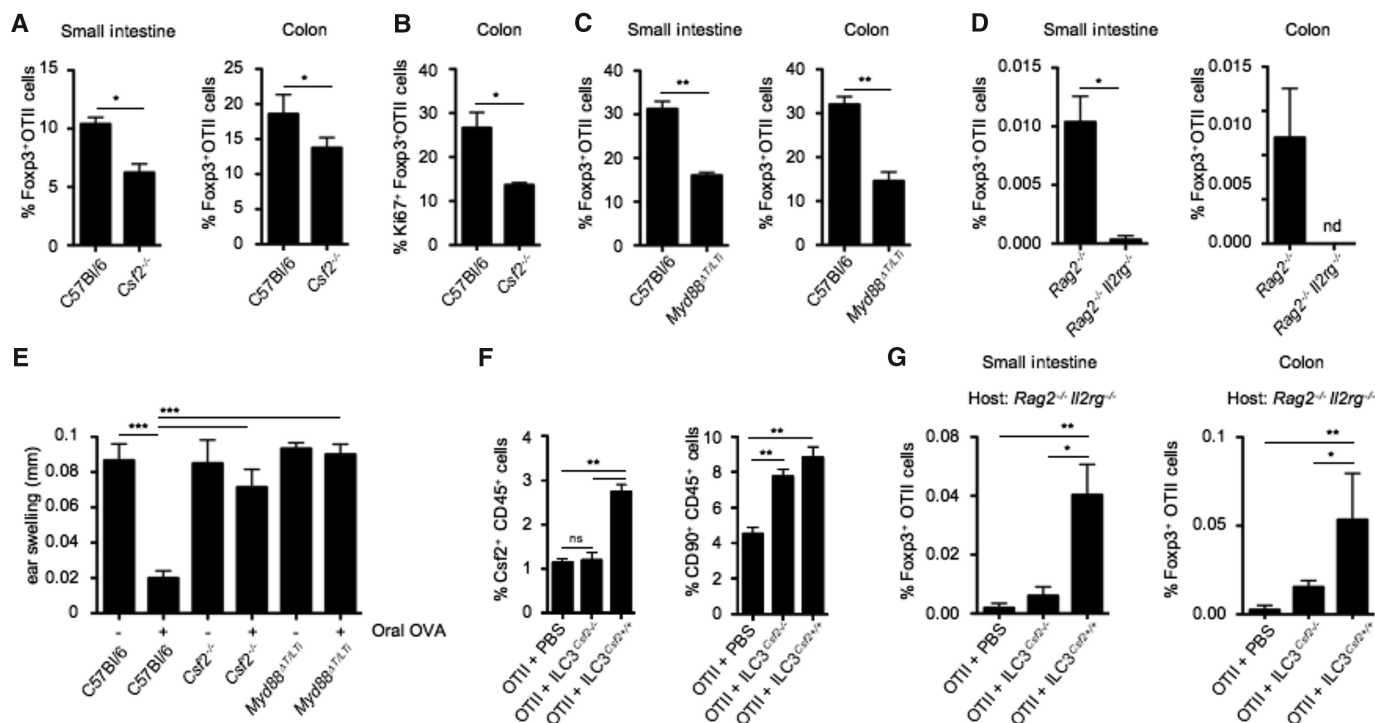


Fig. 4. ILC3-derived Csf2 controls oral tolerance to dietary antigens.

(A) Naïve OTII *Rag2*^{-/-} CD45.1⁺ T cells were adoptively transferred into CD45.2⁺ C57Bl/6 and CD45.2⁺ *Csf2*^{-/-} mice. Bar graph shows percentages of small intestinal and colonic OTII-specific Foxp3⁺ T_{regs} after oral feeding with OVA ad libitum. Data are shown as mean $\pm$ SD of three independent experiments with three mice per group. (B) Bar graph shows percentages of Ki67⁺ colonic Foxp3⁺ OTII T cells in C57Bl/6 and *Csf2*^{-/-} mice after OVA feeding. Data are shown as mean $\pm$ SD of three independent experiments with three mice per experiment. (C and D) Naïve OTII *Rag2*^{-/-} CD45.1⁺ T cells were adoptively transferred into C57Bl/6 and *Myd88* ^{Δ TLT} mice (C), or *Rag2*^{-/-} and *Rag2*^{-/-} *Il2rg*^{-/-} mice (D). Bar graphs show percentages of small intestinal and colonic OTII-specific Foxp3⁺ T_{regs} after OVA feeding. Data are shown as mean $\pm$ SD of three independent experiments with three mice per group. (E) *Csf2*^{-/-}, *Myd88* ^{Δ TLT} and control mice were either fed (+) or not fed with OVA (-) for 7 days to induce oral tolerance. Four days later, fed mice were immunized subcutaneously with

OVA (300 μ g) and complete Freund's adjuvant and rechallenged 14 days later with OVA (50 μ g) into the right ear, as described in the materials and methods. Skin DTH response was determined by ear swelling (mm). Data are shown as mean $\pm$ SD (n = 10 mice) and are representative of two independent experiments. (F and G) Purified wild-type or *Csf2*^{-/-} ILC3 were injected into *Rag2*^{-/-} *Il2rg*^{-/-} hosts, 2 weeks before injection of naïve OTII CD45.1⁺ T cells. Reconstituted hosts were fed with OVA and analyzed 5 days later. (F) Bar graph shows percentages of Csf2⁺ CD45⁺ cells (left) and total CD90⁺ CD45⁺ ILCs (right) in the colonic lamina propria of the indicated host mice. Data are shown as mean $\pm$ SD of two independent experiments with three mice per group. (G) Bar graphs show percentages of small intestinal and colonic OTII-specific Foxp3⁺ T_{regs} after OVA feeding. Data are shown as mean $\pm$ SD of two independent experiments with three mice per group. Student's *t* test (A to D) or one-way ANOVA Bonferroni's multiple comparison test (E to G) were performed. Statistical significance is indicated by **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

specifically in LysM^+ cells [$\text{LysM}^{\text{Cre}};\text{Myd88}^{\text{fllox/fllox}}$ ($\text{Myd88}^{\Delta\text{MP}}$) mice] exhibited a significant reduction in IL-1 β production in intestinal macrophages, whereas IL-1 β production by intestinal DCs was unaffected (Fig. 3F). The disruption of IL-1 β release by intestinal macrophages in $\text{Myd88}^{\Delta\text{MP}}$ mice abrogated the production of Csf2 by $\text{ROR}\gamma^+$ LTi and NCR^+ ILC3 in these animals (Fig. 3G), whereas addition of exogenous IL-1 β cytokine rescued Csf2 production by $\text{ROR}\gamma^+$ ILC3 (Fig. 3G). Consistent with a central role for macrophages in intestinal IL-1 β production, administration of depleting anti-Csf1 receptor monoclonal antibody (anti-Csf1R mAb) depleted tissue macrophages (Fig. 3H), as we previously showed (49), and reduced total *Il1b* expression (Fig. 3I). Accordingly, Csf2 production by intestinal $\text{ROR}\gamma^+$ ILC3 was significantly reduced after depletion of colonic macrophages (Fig. 3J). IL-1 β cytokine was capable of rescuing Csf2 production by $\text{ROR}\gamma^+$ ILC3 isolated from mice treated with the anti-Csf1R mAb (Fig. 3J), thus confirming that macrophage-derived IL-1 β is a key driver of Csf2 production by $\text{ROR}\gamma^+$ ILC3. Myd88 is an essential signal transducer in both the TLR and IL-1R pathway (50). To establish whether IL-1 β and IL-1R signaling are required to promote Csf2 production by $\text{ROR}\gamma^+$ ILC3, we crossed Rorc^{Cre} mice with $\text{Myd88}^{\text{fllox/fllox}}$ mice to achieve deletion of Myd88 specifically in $\text{ROR}\gamma^+$ ILC3 and T cells ($\text{Myd88}^{\Delta\text{TLTi}}$) (51). As expected, we observed a reduction in Csf2 production by $\text{ROR}\gamma^+$ ILC3 from $\text{Myd88}^{\Delta\text{TLTi}}$ mice (Fig. 3K). In this case, administration of IL-1 β cytokine failed to rescue Csf2 expression by $\text{ROR}\gamma^+$ ILC3, suggesting that Myd88 functions downstream of IL-1R in $\text{ROR}\gamma^+$ ILC3 (Fig. 3K). Taken together, our results suggest that Myd88-dependent sensing of the commensal microflora by intestinal macrophages elicits production of IL-1 β , which in turn activates the IL-1R–Myd88 pathway in $\text{ROR}\gamma^+$ ILC3 to drive the steady-state production of Csf2. Alteration of the commensal flora using broad-spectrum antibiotics or deletion of macrophages using anti-Csf1R mAb treatment led to impaired RA production in all DC subsets (fig. S8A) and to reduced T_{reg} numbers and proliferation (fig. S8, B and C), confirming the role of tissue macrophages in translating microbial cues into immunoregulatory signals that help promote T_{reg} homeostasis in the steady-state colon.

Csf2 Promotes Oral Tolerance to Fed Antigens

Because one of the key functions of intestinal T_{reg} s is the maintenance of oral tolerance to fed antigens, we asked whether deficiency in Csf2 affects de novo generation of intestinal T_{reg} s upon oral administration of ovalbumin (OVA). Conversion and expansion of OVA-specific T_{reg} s in the small and large intestine were impaired in $\text{Csf2}^{-/-}$ mice compared to wild-type mice (Fig. 4, A and B). Similar results were obtained when T_{reg} conversion was analyzed in mice selectively lacking Csf2 in $\text{ROR}\gamma^+$ ILC3 ($\text{Myd88}^{\Delta\text{TLTi}}$) or in ILC-deficient mice ($\text{Rag2}^{-/-}\text{Il2rg}^{-/-}$) (Fig. 4, C and D). Consistent with Csf2's key role in oral toler-

ance, OVA feeding of $\text{Csf2}^{-/-}$ mice and $\text{Myd88}^{\Delta\text{TLTi}}$ mice failed to protect the mice from delayed-type hypersensitivity (DTH) reaction upon OVA challenge, whereas control mice were protected (Fig. 4E). Together, these results establish that altered Csf2 production by ILCs impairs the induction of oral tolerance to dietary antigens. The defect in T_{reg} conversion observed in the small intestine of $\text{Csf2}^{-/-}$ mice contrasts with the apparent normal total T_{reg} numbers observed in the small bowel of these mice. These results suggest that compensatory mechanisms specific to the small bowel may help restore the number but likely not the repertoire of small intestinal T_{reg} s in $\text{Csf2}^{-/-}$ mice.

To establish the direct contribution of Csf2 produced by ILC3 to T_{reg} conversion in vivo, we reconstituted ILC-deficient $\text{Rag2}^{-/-}\text{Il2rg}^{-/-}$ mice with $\text{Csf2}^{-/-}$ or $\text{Csf2}^{+/+}$ ILC3. Two weeks later, $\text{Rag2}^{-/-}\text{Il2rg}^{-/-}$ mice reconstituted with ILCs were adoptively transferred with OVA-specific T cell receptor transgenic OTII cells and fed with OVA for 5 days. $\text{Csf2}^{-/-}$ and $\text{Csf2}^{+/+}$ ILC3 engrafted with the same efficiency in $\text{Rag2}^{-/-}\text{Il2rg}^{-/-}$ mice (Fig. 4F), and reconstitution of $\text{Rag2}^{-/-}\text{Il2rg}^{-/-}$ mice with $\text{Csf2}^{+/+}$ ILC3 led to partial recovery of Csf2 production in the intestine (Fig. 4F). Although the rate of T_{reg} conversion was low in all $\text{Rag2}^{-/-}\text{Il2rg}^{-/-}$ mice due to a defect in lymphoid organ development in these mice, OVA-specific T_{reg} conversion was nonetheless significantly higher in $\text{Rag2}^{-/-}\text{Il2rg}^{-/-}$ mice reconstituted with $\text{Csf2}^{+/+}$ compared to mice reconstituted with $\text{Csf2}^{-/-}$ ILC3 (Fig. 4G), further emphasizing the contribution of ILC3-derived Csf2 to the control of oral tolerance to dietary antigens.

Discussion

Previous studies have established the role of microbial commensals that colonize the large bowel to promote the induction of Foxp3 $^+$ T_{reg} differentiation (5). However the cellular cues that promote T_{reg} accumulation in response to gut commensals have only recently started to be unraveled (52–54). Our data identify a mechanism by which the gut microbiota promotes intestinal homeostasis by supporting a crosstalk between IL-1 β -secreting macrophages and Csf2-producing $\text{ROR}\gamma^+$ ILC3 in the intestinal mucosa. Microbiota-driven IL-1 β production by macrophages promoted the release of Csf2 by ILC3, which in turn acted on DCs and macrophages, allowing for the maintenance of colonic T_{reg} homeostasis (fig. S9). Ablation of Csf2 altered DC and macrophage numbers and impaired their ability to produce regulatory factors such as RA and IL-10, which led to disrupted T_{reg} homeostasis in the large intestine. Conversely, administration of Csf2 cytokine increased T_{reg} frequency in the gut. Most notably, cell type-specific ablation of IL-1-dependent signaling in $\text{ROR}\gamma^+$ ILC3 abrogated oral tolerance to dietary antigens and compromised intestinal T_{reg} homeostasis in vivo. Although the reduction in total T_{reg} numbers was mostly observed in the large intestine, adoptive transfer studies in $\text{Csf2}^{-/-}$ mice revealed impaired T_{reg} differentiation both in the small and large intestine, suggesting that Csf2-

dependent MNP immunoregulatory functions control T_{reg} induction in both tissues

Establishing intestinal tolerance is critical for the prevention of intestinal diseases such as IBD. IBD includes two broad disease classifications known as ulcerative colitis and Crohn's disease, but there is substantial variation in IBD clinicopathology in individual patients; hence, it is likely that numerous subtypes of IBD exist in this group. In a study of more than 300 patients with Crohn's disease, the presence of neutralizing antibodies to Csf2 in the serum correlated with ileal involvement and the development of penetrating pathology, whereas a more recent study identified reduced levels of Csf2 receptor (Csf2R) and impaired receptor activity in a mixed group of IBD patients (55, 56). Previous clinical trials of recombinant Csf2 in IBD have established patient benefit in terms of reduced disease severity and lower burden of corticosteroid use (57). Unpublished results of a larger trial of Csf2 in IBD has since failed to achieve primary clinical end points, but it remains likely that a subset of IBD patients with defective Csf2 production or function could benefit from this therapy.

The uncovered key role for Csf2 in the maintenance of intestinal tolerance is consistent with previous studies showing that absence of Csf2 can also contribute to lupus-like disease, insulinitis, and age-related glucose intolerance (29, 58) and further emphasizes the critical role of tissue-resident phagocytes in the maintenance of tissue integrity.

Our data reveal a mechanism by which the gut commensal flora promotes immune homeostasis in the host. We have identified the commensal-driven MNP-ILC-Csf2 axis as a key regulator of intestinal T cell homeostasis in the mouse intestine. Disturbance of this axis radically altered MNP effector function, resulting in impaired oral tolerance to dietary antigens. These results represent an important advance in our understanding of how commensal microbes can regulate host intestinal immunity and may inform the design of new immunotherapies for the use in patients with subtypes of IBD.

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Supplementary Materials

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Early Childhood Investments Substantially Boost Adult Health

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High-quality early childhood programs have been shown to have substantial benefits in reducing crime, raising earnings, and promoting education. Much less is known about their benefits for adult health. We report on the long-term health effects of one of the oldest and most heavily cited early childhood interventions with long-term follow-up evaluated by the method of randomization: the Carolina Abecedarian Project (ABC). Using recently collected biomedical data, we find that disadvantaged children randomly assigned to treatment have significantly lower prevalence of risk factors for cardiovascular and metabolic diseases in their mid-30s. The evidence is especially strong for males. The mean systolic blood pressure among the control males is 143 millimeters of mercury (mm Hg), whereas it is only 126 mm Hg among the treated. One in four males in the control group is affected by metabolic syndrome, whereas none in the treatment group are affected. To reach these conclusions, we address several statistical challenges. We use exact permutation tests to account for small sample sizes and conduct a parallel bootstrap confidence interval analysis to confirm the permutation analysis. We adjust inference to account for the multiple hypotheses tested and for nonrandom attrition. Our evidence shows the potential of early life interventions for preventing disease and promoting health.

Noncommunicable diseases are responsible for roughly two-thirds of worldwide deaths (1). Most policies that combat disease currently focus on treatment after disease occurs and on reducing risk factors in adult life. Recent discussions of effective ways of controlling the soaring costs of the U.S. health care system emphasize tertiary prevention—that is, reducing the worsening of the conditions of those already ill [see, e.g., (2)] and “bending the cost curve” for such treatments (2–5).

A complementary approach is to prevent disease or to delay its onset. A large body of evidence shows that adult illnesses are more prevalent and problematic among those who have experienced adverse early life conditions (6, 7). The exact mechanisms through which early life experiences translate into later life health are being actively investigated (8, 9).

This paper shows that high-quality, intensive interventions in the early years can be effective in preventing, or at least delaying, the onset of adult disease. The recent literature establishes that interventions that enrich the environments of disadvantaged children have substantial impacts on a variety of outcomes throughout their lives [see, e.g., (10–12)]. However, little is known about their benefits on health [see, e.g., (13)].

We study the long-term health effects of one of the oldest and most cited early childhood programs: the Carolina Abecedarian Project (ABC).

ABC was designed as a social experiment to investigate whether a stimulating early childhood environment could prevent the development of mild mental retardation in disadvantaged children. The study was conducted on four cohorts of disadvantaged children born between 1972 and 1977 who were living in or near Chapel Hill, North Carolina. The base sample included 109 families (111 children). Of these 111 children, 57 were assigned to treatment status and 54 were assigned to control status. The intervention consisted of a two-stage treatment targeted to different segments of child life cycles: an early childhood stage (from birth through age 5) and a subsequent school-age stage (from age 6 through 8). The first stage of the intervention involved periods of cognitive and social stimulation interspersed with caregiving and supervised play throughout a full 8-hour day for the first 5 years. The stimulation component was based on a curriculum that emphasized development of language, emotional regulation, and cognitive skills (14, 15). The second stage of the intervention focused on improving early math and reading skills through having “home-school resource teachers” customize learning activities based on materials being covered at school and then deliver these materials to the parents to use at home. The treatment and control groups from the first stage were randomly assigned to treatment and control groups in the second stage. We analyzed data on treatment and control groups created by the first-stage randomization. We found no evidence of any treatment effect on adult health from the second-stage randomization. The treatment effects are much smaller in magnitude than those estimated for the first-stage treatment and fail to achieve statistical significance at conventional levels. See the supplementary materials,

section F, for evidence on this issue. References (16–18) show that for most outcomes the early educational intervention had much stronger effects than the school-age treatment. Additionally, previous work has also shown no health effects from a school-age (as compared with a preschool) educational intervention (19). The available evidence on interventions to prevent obesity points to the years 0 through 5 as a critical period (as compared with after 5 years) [see, e.g., (20–22)].

The ABC intervention also had a nutritional and health care component during the first stage. Treated children had two meals and a snack at the childcare center. They were offered primary pediatric care (both well- and ill-child care), with periodic check-ups and daily screening. More details on the intervention are given in the supplementary materials, section A.

Data

Data were collected on both treated and control cases from the beginning of their participation in the study, using surveys administered to children, parents, and teachers, as well as direct assessments. Before the intervention started, baseline information was gathered on parental characteristics, family structure, socioeconomic status, and birth circumstances. For both treated and control cases, data on cognition, personality, health, achievement, and behavior were then collected at multiple stages from birth until the end of school-age treatment. At the end of the second stage of treatment, participants were followed up at ages 12, 15, 21, 30, and in the mid-30s. Details on the outcomes and covariates used in this analysis are provided in the supplementary materials, section B.

A biomedical survey of cardiovascular and metabolic risk factors was conducted when participants were in their mid-30s. Information on biomeasures was collected from two sources. The first source was a physical exam carried out by a local physician in the Chapel Hill Internal Medicine practice, in which the same doctor (blind to treatment status) examined all subjects. In this exam, measurements were collected on weight (pounds), height (inches), waist (inches), hips (inches), and systolic and diastolic blood pressure (bp). The physician also checked the status of several body systems. The physician carried out a complete physical exam and checked whether there was abnormality in relation to the following systems: skin, HEENT (head, ear, eye, nose, and throat), neck, chest, lung, breast, cardiovascular, abdomen, neurologic, muscle strength and tone, musculoskeletal, and lymphatic. The second source was laboratory tests, based on nonfasting venous blood collected from the subjects during the medical visit (the phlebotomist was blind to treatment status, and the blood samples were sent out to another facility for analysis and report preparation).

Several issues arise in evaluating the health effects of the ABC intervention. First, the sample size is small. Conventional testing approaches that rely on large-sample properties of test statistics

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may be inappropriate. To surmount this problem, we use exact (small-sample) block permutation tests. We show in tables S25 and S26 that when we use bootstrap methods that have a large sample justification, we obtain the same inference about treatment effects. Bootstrapping has the additional benefit of producing confidence intervals to gauge the uncertainty inherent in our estimates.

Second, numerous treatment effects are analyzed. This creates an opportunity for “cherry picking”—finding spurious treatment effects merely by chance if conventional one-hypothesis-at-a-time approaches to testing are used. We account for the multiplicity of the hypotheses being tested using recently developed stepdown procedures (23).

Third, information is missing due to nonrandom attrition from the survey, potentially undermining the validity of inference. We investigate

the causes of missing information and correct for potential bias using inverse probability weighting (IPW) (24, 25). More information on the methodology and a detailed analysis of the attrition patterns is presented in the supplementary materials, sections C, D, and H.

Results

Physical Health

Estimated treatment effects and associated test statistics are given in Tables 1 (males) and 2 (females). Throughout the paper, we report one-sided single-hypothesis block permutation *P* values associated with the IPW treatment effect estimates; multiple hypothesis stepdown *P* values are reported in Tables 1 and 2. We first report the experimental results on the biomarkers of cardiovascular functioning. On average, treated males have lower

values of both systolic and diastolic bp. This difference amounts to 13.5 mm Hg for diastolic bp ($P = 0.024$) and 17.5 mm Hg for systolic bp ($P = 0.018$). Treated females are less likely to be prehypertensive. The prevalence of prehypertension (systolic bp ≥ 120 or diastolic bp ≥ 80) (26) is 0.909 in the control group and 0.667 in the treatment group, and the difference is statistically significant ($P = 0.042$). Using two different definitions of hypertension [systolic bp ≥ 140 and diastolic bp ≥ 90 (27) and systolic bp ≥ 140 or diastolic bp ≥ 90 (26)], treated males are less likely to fall into the stage I hypertension category (a prevalence of only 0.105 or 0.211) as compared with a much higher prevalence observed in the control group (0.444 and 0.556). Both treatment effects are statistically significant ($P = 0.010$ and $P = 0.038$) (28).

Table 1. ABC intervention, males: main health results, biomedical sweep. This table presents the inference and descriptive statistics of selected outcomes of the ABC intervention. The first column describes the outcome analyzed. The remaining six columns present the statistical analysis. The columns present the following information: (i) Control mean. (ii) Treatment mean. (iii) Unconditional difference in means across treatment and control groups. We multiply the difference in means by (–1) when a higher value of the variable in the raw data represents a worse outcome so that all outcomes are normalized in a favorable direction (but are not restricted to be positive). (iv) Conditional treatment effect controlling for cohort, number of siblings, mother’s IQ, and high-risk index at birth, and accounting for attrition using IPW. Probabilities of IPW are estimated using the following variables: prematurity (gestational age < 37 weeks), a dichotomous indicator for not having an exam for illness or injury in the past 2 years at age 30, Achenbach DSM attention-deficit/hyperactivity (AD/H) problems scale at age 30, and

Achenbach substance abuse scale at age 30. The selection of covariates for IPW is based on the lowest Akaike Information Criteria (AIC) among models examining all combinations of covariates that present statistically significant imbalance between attriters and nonattriters. See supplementary materials section C and table S1 for details. (v) One-sided single-hypothesis block permutation *P* value associated with the IPW treatment effect estimate. By block permutation, we mean that permutations are done within strata defined by the preprogram variables used in the randomization protocol: cohort, gender, number of siblings, mother’s IQ, and high-risk index. (vi) Multiple hypothesis stepdown *P* values associated with (v). The multiple hypothesis testing is applied to blocks of outcomes. Blocks of variables that are tested jointly using the stepdown algorithm are delineated by horizontal lines. *P* values ≤ 0.10 are in bold type. HbA1C, glycosylated hemoglobin; NCEP, National Cholesterol Education Program. See table S11 for complete estimation results.

Variable	Control mean	Treatment mean	Difference in means	Conditional treatment effect	Block <i>P</i> value	Stepdown <i>P</i> value
<i>Blood pressure</i>						
Diastolic blood pressure (mm Hg)	92.000	78.526	13.474	19.220	0.024	0.024
Systolic blood pressure (mm Hg)	143.333	125.789	17.544	24.828	0.018	0.029
Prehypertension (systolic bp ≥ 120 and diastolic bp ≥ 80)	0.667	0.421	0.246	0.321	0.119	0.172
Prehypertension (systolic bp ≥ 120 or diastolic bp ≥ 80)	0.778	0.684	0.094	0.096	0.235	0.235
Hypertension (systolic bp ≥ 140 and diastolic bp ≥ 90)	0.444	0.105	0.339	0.537	0.010	0.018
Hypertension (systolic bp ≥ 140 or diastolic bp ≥ 90)	0.556	0.211	0.345	0.404	0.038	0.038
<i>Laboratory tests</i>						
High-density lipoprotein (HDL) cholesterol (mg/dL)	42.000	53.211	11.211	11.720	0.066	0.110
Dyslipidemia (HDL < 40 mg/dL)	0.417	0.106	0.311	0.255	0.179	0.179
Prediabetes (HbA1C $\geq 5.7\%$)	0.583	0.473	0.110	0.043	0.426	0.426
Vitamin D deficiency (<20 ng/mL)	0.750	0.368	0.382	0.435	0.021	0.021
<i>Obesity</i>						
Overweight (BMI ≥ 25)	0.750	0.722	0.028	0.190	0.239	0.239
Obese (BMI ≥ 30)	0.625	0.556	0.069	0.211	0.233	0.345
Severely obese (BMI ≥ 35)	0.375	0.111	0.264	0.404	0.115	0.232
Waist-hip ratio (WHR)	0.962	0.937	0.025	0.045	0.293	0.293
Abdominal obesity (WHR > 0.9)	0.875	0.647	0.228	0.294	0.137	0.218
<i>Multiple risk factors</i>						
Obesity and hypertension	0.500	0.111	0.389	0.529	0.016	0.016
Severe obesity and hypertension	0.375	0.000	0.375	0.502	0.005	0.012
Hypertension and dyslipidemia	0.333	0.000	0.333	0.435	0.006	0.012
Metabolic syndrome (NCEP definition)	0.250	0.000	0.250	0.465	0.007	0.014
Framingham risk score (34)	7.043	4.889	2.154	3.253	0.038	0.038

Biomarkers of metabolic activity from blood tests (lipid panel) show that treated individuals have higher levels of high-density lipoprotein cholesterol (HDL-C)—“good” cholesterol. The magnitude of the difference between treated and control groups is larger for males. The control males have a level of HDL cholesterol of 42 mg/dL, which is just above the lower recommended limit of 40 mg/dL (29), whereas the level for the treated males is 11 mg/dL higher. The treatment effect is marginally significant ($P = 0.066$). This is reflected in the prevalence of dyslipidemia (elevated lipid levels). The difference in the prevalence of this condition between treatment and control groups is 0.311 for males (HDL-C < 40 mg/dL; $P = 0.179$) and 0.177 for females (HDL-C < 50 mg/dL; $P = 0.099$). The healthier metabolic status experienced by the male treatment group is confirmed by the lower prevalence of pre-

diabetes indicators [glycosylated hemoglobin $\geq 5.7\%$ (30), 0.473 versus 0.583], although the difference does not attain statistical significance ($P = 0.426$). Control males are also twice as likely to be affected by vitamin D deficiency (total vitamin D < 20 ng/mL (31); 0.368 versus 0.750; $P = 0.021$). The prevalence of both severe and abdominal obesity is lower among treatment group males but the differences are not statistically significant at the 10% level. Treated females are less likely than controls to be affected by abdominal obesity, both when considering the waist-hip ratio (WHR) and when analyzing a dichotomous measure of WHR > 0.85 (32) (0.563 versus 0.762); both treatment effects are marginally significant ($P = 0.063$ and $P = 0.080$, respectively). The health effects of the ABC intervention translate into lower prevalence of multiple risk

factors that are particularly striking for males. Those in the treatment group are less likely to experience both obesity and hypertension [difference in mean (diff.) = 0.389; $P = 0.016$], severe obesity and hypertension (diff. = 0.375; $P = 0.005$), and dyslipidemia and hypertension (diff. = 0.333; $P = 0.006$). None of the treated males have the cluster of conditions known as metabolic syndrome [defined as waist circumference > 102 cm or 40 inches (33); HDL-C < 40 mg/dL; bp $\geq 130/85$ mm Hg (29)], associated with greater risk of heart disease, stroke, and diabetes, whereas one in four in the control group is affected by it ($P = 0.007$). The prevalence of the metabolic syndrome for females [defined as waist circumference > 88 cm or 35 inches (33); HDL-C < 50 mg/dL; bp $\geq 130/85$ mm Hg (29)] is lower in the treatment group, but the differences are not statistically significant at the 10% level. Finally, results for the Framingham

Table 2. ABC intervention, females: main health results, biomedical sweep. This table presents the inference and descriptive statistics of selected outcomes of the ABC intervention. The first column describes the outcome analyzed. The remaining six columns present the statistical analysis. The columns present the following information: (i) Control mean. (ii) Treatment mean. (iii) Unconditional difference in means across treatment and control groups. We multiply the difference in means by (–1) when a higher value of the variable in the raw data represents a worse outcome so that all outcomes are normalized in a favorable direction (but are not restricted to be positive). (iv) Conditional treatment effect controlling for cohort, number of siblings, mother’s IQ, and high-risk index at birth, and accounting for attrition using IPW. Probabilities of IPW are estimated using the following variables for the biomedical sweep outcomes: prematurity (gestational age <37 weeks), mother Wechsler Adult Intelligence Scale (WAIS) digit symbol score at recruitment,

Achenbach rule-breaking problem scale at age 30, and Achenbach substance abuse scale at age 30. The selection of covariates for IPW is based on the lowest AIC among models examining all combinations of covariates that present statistically significant imbalance between attriters and non-attriters. See supplementary materials section C and table S2 for details. (v) One-sided single-hypothesis block permutation P value associated with the IPW treatment effect estimate. By block permutation, we mean that permutations are done within strata defined by the preprogram variables used in the randomization protocol: cohort, gender, number of siblings, mother’s IQ, and high-risk index. (vi) Multiple hypothesis stepdown P values associated with (v). The multiple hypothesis testing is applied to blocks of outcomes. Blocks of variables that are tested jointly using the stepdown algorithm are delineated by horizontal lines. P values ≤ 0.10 are in bold type. See table S12 for complete estimation results.

Variable	Control mean	Treatment mean	Difference in means	Conditional treatment effect	Block P value	Stepdown P value
<i>Blood pressure</i>						
Diastolic blood pressure (mm Hg)	89.227	85.333	3.894	1.204	0.446	0.446
Systolic blood pressure (mm Hg)	135.636	129.666	5.970	2.185	0.300	0.380
Prehypertension (systolic bp ≥ 120 and diastolic bp ≥ 80)	0.727	0.500	0.227	0.101	0.222	0.222
Prehypertension (systolic bp ≥ 120 or diastolic bp ≥ 80)	0.909	0.667	0.242	0.244	0.042	0.069
Hypertension (systolic bp ≥ 140 and diastolic bp ≥ 90)	0.318	0.222	0.096	–0.003	0.375	0.499
Hypertension (systolic bp ≥ 140 or diastolic bp ≥ 90)	0.409	0.500	–0.091	–0.181	0.721	0.721
<i>Laboratory tests</i>						
High-density lipoprotein (HDL) cholesterol (mg/dL)	55.318	60.444	5.126	6.002	0.143	0.143
Dyslipidemia (HDL < 50 mg/dL)	0.455	0.278	0.177	0.201	0.099	0.147
Prediabetes (HbA1C $\geq 5.7\%$)	0.364	0.353	0.011	0.070	0.580	0.580
Vitamin D deficiency (<20 ng/mL)	0.727	0.722	0.005	0.048	0.303	0.303
<i>Obesity</i>						
Overweight (BMI ≥ 25)	0.955	0.889	0.066	0.054	0.482	0.690
Obese (BMI ≥ 30)	0.727	0.666	0.061	–0.112	0.790	0.790
Severely obese (BMI ≥ 35)	0.364	0.223	0.141	0.143	0.354	0.653
Waist-hip ratio (WHR)	0.933	0.876	0.057	0.053	0.063	0.101
Abdominal obesity (WHR > 0.85)	0.762	0.563	0.199	0.198	0.080	0.080
<i>Multiple risk factors</i>						
Obesity and hypertension	0.364	0.278	0.086	–0.028	0.501	0.641
Severe obesity and hypertension	0.136	0.167	–0.031	–0.066	0.696	0.696
Hypertension and dyslipidemia	0.182	0.167	0.015	–0.043	0.486	0.725
Metabolic syndrome (NCEP definition)	0.190	0.062	0.128	0.057	0.184	0.393
Framingham risk score (34)	1.482	1.143	0.339	0.331	0.070	0.070

risk score (34) reveal that both treated males and females have a significantly lower risk of experiencing “total” coronary heart disease (CHD), defined as both stable and unstable angina, myocardial infarction, or CHD death, within the next 10 years (diff. = 2.154, $P = 0.038$ for males; diff. = 0.339, $P = 0.070$ for females).

In sum, the available evidence from the biomedical survey of ABC shows that the children who attended the child care center in the first 5 years of their lives enjoy better physical health in their mid-30s, with significant markers indicating better future health. The benefits of these health improvements are substantial and wide-ranging. Reference (35) provides a detailed review of the labor market costs of obesity, which range from increased absenteeism to lower productivity and wages. There are considerable losses in life expectancy due to obesity. Reference (36) reports estimates that 35-year-old males with hypertension would gain 1.1 to 5.3 years of expected life (0.9 to 5.7 years for females) from reducing their diastolic bp to 88 mm Hg using the Coronary Heart Disease Policy Model based on data from the Framingham Heart Study. Reference (37), using data from the Framingham Heart Study, finds that 40-year-old male nonsmokers suffer a loss of life expectancy of 3.1 years (3.3 years for females) because of being overweight, and of

5.8 years (7.1 years for females) because of obesity. Reference (38), using data from the National Longitudinal Study of Adolescent Health, shows that diabetics are less likely to be employed (by 8 to 11 percentage points), are more likely to participate in social programs (by 8 to 13 percentage points), and earn on average lower wages (by \$1500 to \$6000). Reference (39) provides further evidence from the National Longitudinal Survey of Youth 1979 that the duration of diabetes is negatively associated with employment and wages. Reference (40) reports a hazard ratio of 1.47 (95% confidence interval of 1.13 to 1.92) for all-cause mortality and of 2.53 (95% CI of 1.74 to 3.67) for cardiovascular mortality caused by metabolic syndrome (NCEP definition) in the San Antonio Heart Study.

Health Care

Availability of health care is a necessary condition for enjoying better health, although not a sufficient one (41). The upper panel of Table 3 reveals that treated males were more likely to be covered by health insurance at age 30 (0.704 versus 0.476; $P = 0.039$) and to be cared for in a hospital or by a doctor when sick (0.815 versus 0.524; $P = 0.037$). There are no significant differences in the effect of the treatment for females (upper panel of Table 4).

Physical Development

We analyze the effects of the intervention on early physical development, assessed using anthropometric measurements (height and weight) taken when the children had their routine assessments at multiple times in childhood. We transform the body mass index (BMI) measures into standard normal variates (z scores) using the lambda-mu-sigma (LMS) method developed in (42–44). The results are reported in the bottom panel of Table 3. Treated males were less likely than controls to be overweight throughout their preschool years, with almost no treated child having a weight-for-length above the 85th percentile [the age-specific measure for being “at-risk overweight” (45)] in the first 2 years of life. Control males had a greater weight-for-length z -score change between birth and 24 months of age. More rapid increases in weight-for-length in the first 6 months of life have been associated with increased risk of obesity at age 3 (46). Looking at the full BMI distribution by treatment status for males shown in Fig. 1, it is evident that the distribution is both less spread out and shifted to the left for treated males relative to controls. These results are consistent with the obesity-reducing effects found in Head Start (47, 48) and are consistent with evidence in the literature of the important role played by early-life nutrition (49). Further evidence on the

Table 3. ABC intervention, males: health care at age 30; physical development in childhood. This table presents the inference and descriptive statistics of selected outcomes of the ABC intervention. The first column describes the outcome analyzed. The remaining six columns present the statistical analysis. The columns present the following information: (i) Control mean. (ii) Treatment mean. (iii) Unconditional difference in means across treatment and control groups. We multiply the difference in means by (–1) when a higher value of the variable in the raw data represents a worse outcome so that all outcomes are normalized in a favorable direction (but are not restricted to be positive). (iv) Conditional treatment effect controlling for cohort, number of siblings, mother’s IQ, and high-risk index at birth, and accounting for attrition using IPW. The selection of covariates for IPW is based on the lowest AIC among models examining all combinations of covariates that present statistically significant imbalance between attriters and non-

attriters. See supplementary materials section C and table S1 for details. (v) One-sided single-hypothesis block permutation P value associated with the IPW treatment effect estimate. By block permutation, we mean that permutations are done within strata defined by the preprogram variables used in the randomization protocol: cohort, gender, number of siblings, mother’s IQ, and high-risk index. (vi) Multiple hypothesis stepdown P values associated with (v). The multiple hypothesis testing is applied to blocks of outcomes. Blocks of variables that are tested jointly using the stepdown algorithm are delineated by horizontal lines. P values ≤ 0.10 are in bold type. CDC, Centers for Disease Control and Prevention. WHO, World Health Organization. We use weight-for-length ≥ 85 th percentile for being “at-risk overweight” under 24 months and BMI-for-age ≥ 85 th percentile for being overweight for 24 months and older (45). See table S13 for complete estimation results.

Variable	Control mean	Treatment mean	Difference in means	Conditional treatment effect	Block P value	Stepdown P value
<i>Health care at age 30</i>						
Health insurance coverage at age 30	0.476	0.704	0.228	0.226	0.039	0.039
Buys health insurance at age 30	0.333	0.630	0.296	0.248	0.035	0.080
Hospital or doctor office care when sick at age 30	0.524	0.815	0.291	0.265	0.037	0.068
<i>Physical development in childhood</i>						
At risk overweight (CDC) at 3 months	0.227	0.037	0.190	0.206	0.026	0.121
At risk overweight (CDC) at 6 months	0.250	0.080	0.170	0.205	0.074	0.182
At risk overweight (CDC) at 9 months	0.412	0.000	0.412	0.446	0.004	0.023
At risk overweight (CDC) at 12 months	0.429	0.000	0.429	0.408	0.001	0.009
At risk overweight (CDC) at 18 months	0.389	0.000	0.389	0.385	0.000	0.004
Overweight (CDC) at 24 months	0.333	0.000	0.333	0.343	0.001	0.011
Overweight (CDC) at 36 months	0.158	0.080	0.078	0.094	0.194	0.194
Overweight (CDC) at 48 months	0.300	0.167	0.133	0.133	0.150	0.235
Overweight (CDC) at 60 months	0.300	0.125	0.175	0.187	0.058	0.179
Overweight (CDC) at 96 months	0.421	0.120	0.301	0.286	0.030	0.117
Weight-for-length change 0–24 months (CDC)	0.858	–0.105	0.963	1.176	0.058	0.058
Weight-for-length change 0–24 months (WHO)	1.265	0.166	1.100	1.397	0.049	0.057

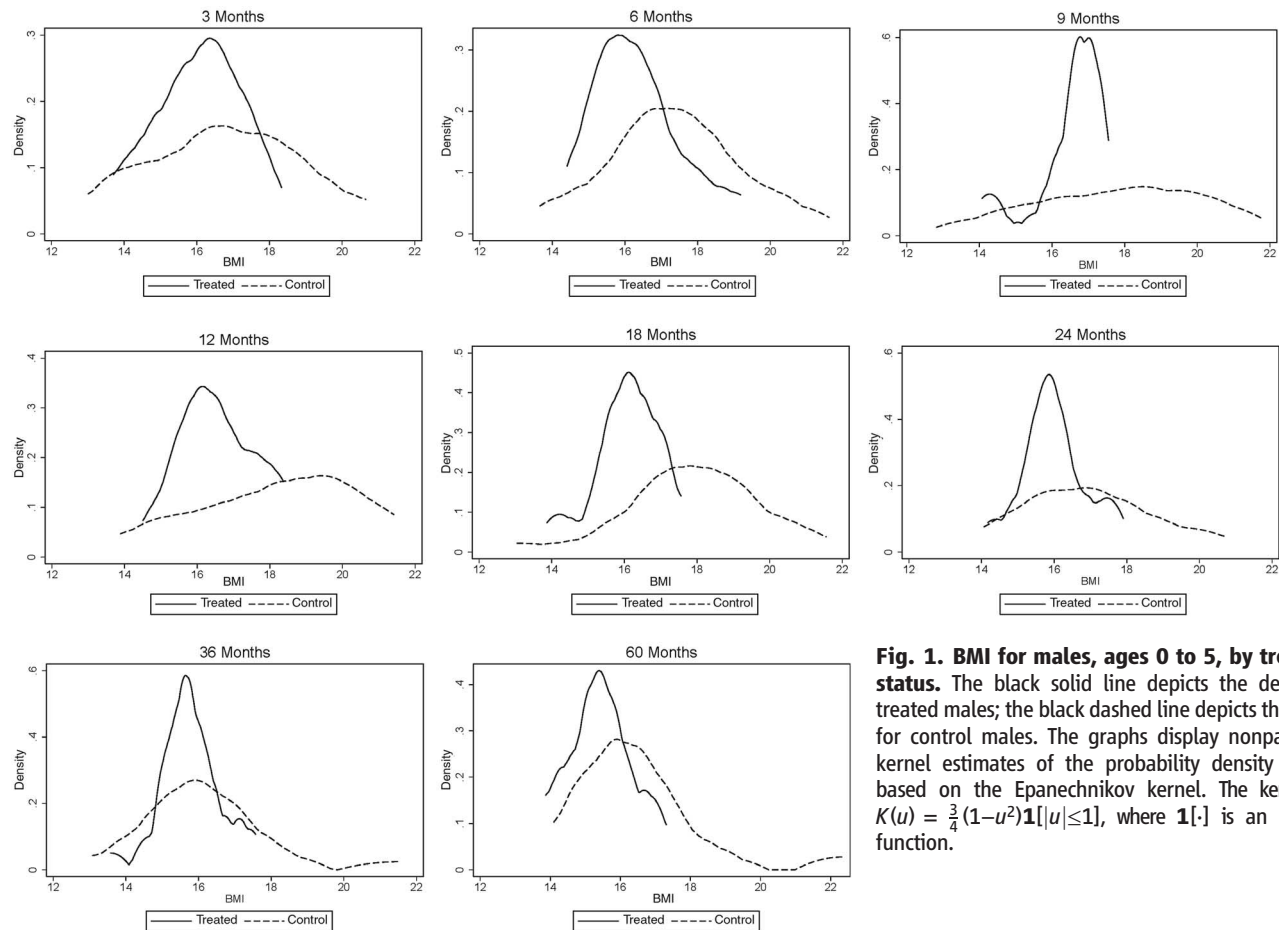


Fig. 1. BMI for males, ages 0 to 5, by treatment status. The black solid line depicts the density for treated males; the black dashed line depicts the density for control males. The graphs display nonparametric kernel estimates of the probability density function based on the Epanechnikov kernel. The kernel K is $K(u) = \frac{3}{4}(1-u^2)\mathbf{1}[|u|\leq 1]$, where $\mathbf{1}[\cdot]$ is an indicator function.

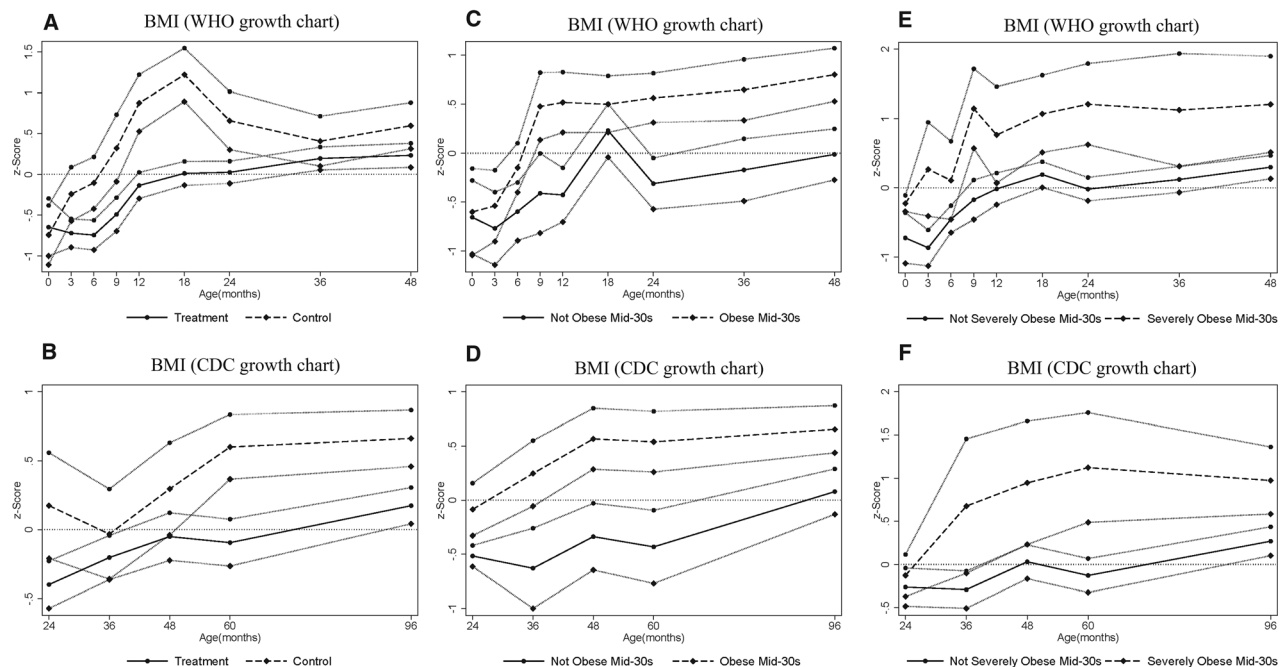


Fig. 2. BMI for males ages 0 to 4 years (A, C, and E) and 2 to 8 years (B, D, and F), by treatment and obesity status at mid-30s. The graphs show BMI z scores at different points in childhood (0, 3, 6, 9, 12, 18, 24, 36, 48, 60, and 96 months) by treatment and control status [(A) and (B)], by obesity status (BMI ≥ 30) in adulthood [(C) and (D)], and by severe obesity status (BMI ≥ 35) in adulthood [(E) and (F)]. Solid and dashed

lines represent mean BMI by age for different groups, and the bands around each line represent standard errors for the corresponding means (one standard error above and below). (A), (C), and (E) use the WHO growth charts to construct the z scores; (B), (D), and (F) use the CDC growth charts. The CDC recommends the use of the WHO growth charts for less than 2 years of age (see www.cdc.gov/growthcharts/who_charts.htm).

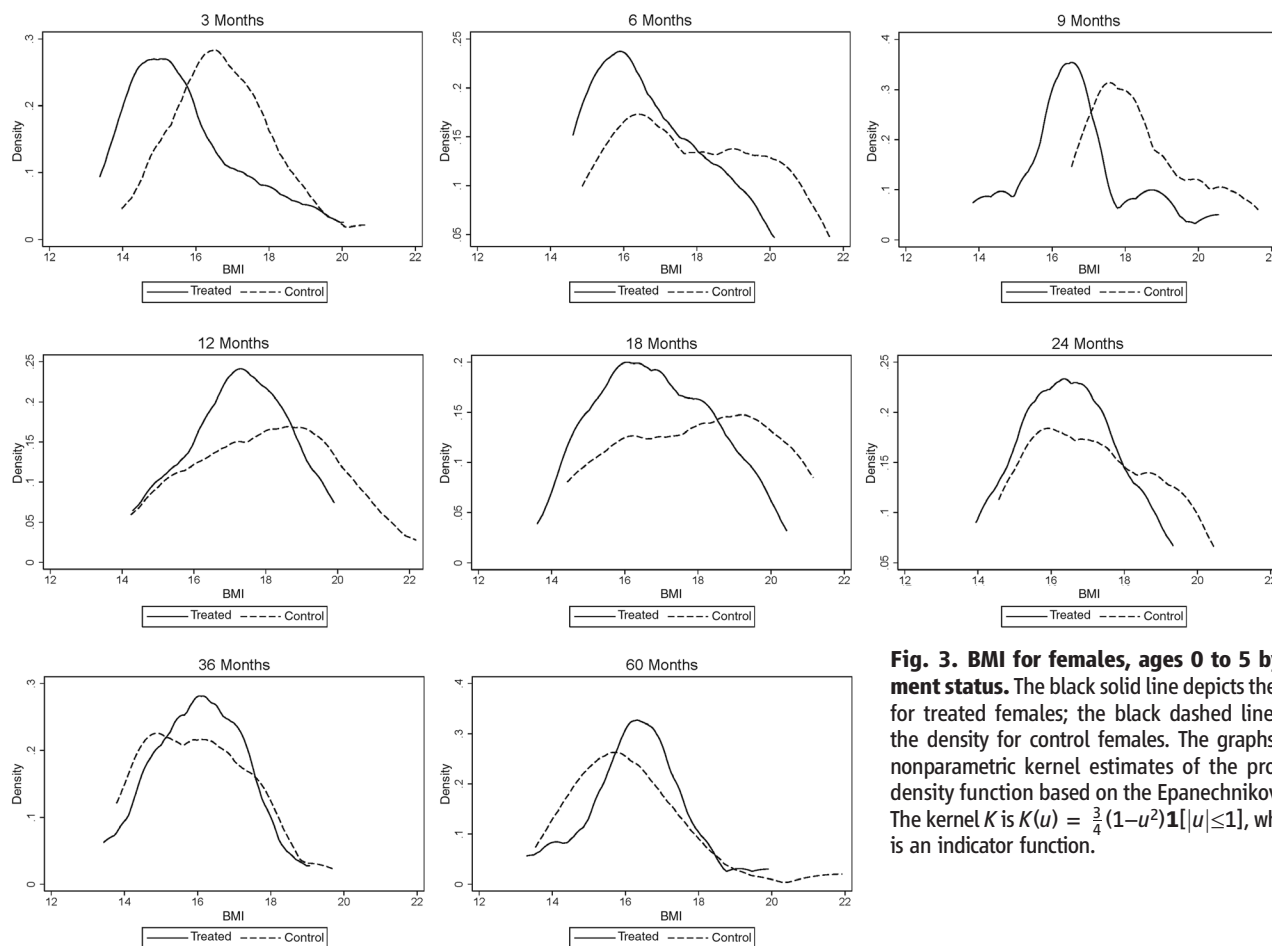


Fig. 3. BMI for females, ages 0 to 5 by treatment status. The black solid line depicts the density for treated females; the black dashed line depicts the density for control females. The graphs display nonparametric kernel estimates of the probability density function based on the Epanechnikov kernel. The kernel K is $K(u) = \frac{3}{4}(1-u^2)\mathbf{1}[|u| \leq 1]$, where $\mathbf{1}[\cdot]$ is an indicator function.

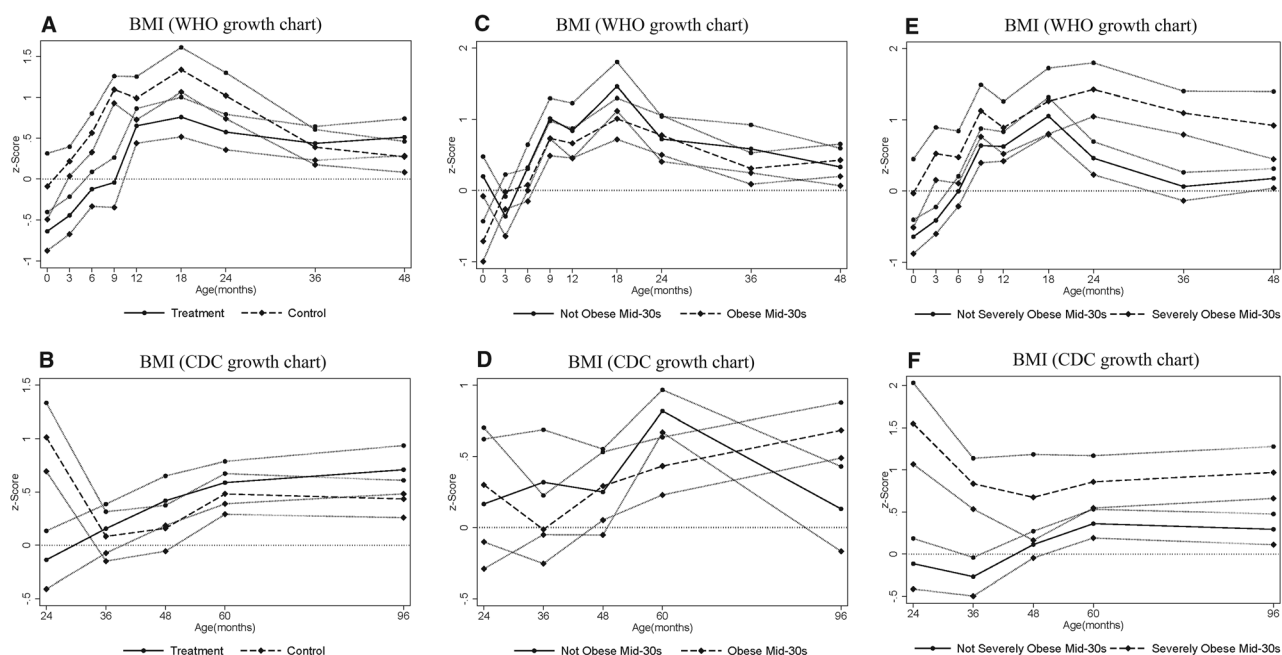


Fig. 4. BMI for females ages 0 to 4 years (A, C, and E) and 2 to 8 years (B, D, and F), by treatment and obesity status at mid-30s. The graphs show BMI z scores at different points in childhood (0, 3, 6, 9, 12, 18, 24, 36, 48, 60, and 96 months) by treatment and control status [(A) and (B)], by obesity status (BMI ≥ 30) in adulthood [(C) and (D)], and by severe obesity status (BMI ≥ 35) in adulthood [(E) and (F)]. Solid and dashed lines

represent mean BMI by age for different groups, and the bands around each line represent standard errors for the corresponding means (one standard error above and below). (A), (C), and (E) use the WHO growth charts to construct the z scores; (B), (D), and (F) use the CDC growth charts. The CDC recommends the use of the WHO growth charts for less than 2 years of age (see www.cdc.gov/growthcharts/who_charts.htm).

Table 4. ABC intervention, females: health care at age 30; physical development in childhood. This table presents the inference and descriptive statistics of selected outcomes of the ABC intervention. The first column describes the outcome analyzed. The remaining six columns present the statistical analysis. The columns present the following information: (i) Control mean. (ii) Treatment mean. (iii) Unconditional difference in means across treatment and control groups. We multiply the difference in means by (–1) when a higher value of the variable in the raw data represents a worse outcome so that all outcomes are normalized in a favorable direction (but are not restricted to be positive). (iv) Conditional treatment effect controlling for cohort, number of siblings, mother’s IQ, and high-risk index at birth, and accounting for attrition using IPW. The selection of covariates for IPW is based on the lowest AIC among models examining all combinations of covariates that present sta-

tistically significant imbalance between attriters and nonattriters. See supplementary materials section C and table S2. (v) One-sided single-hypothesis block permutation *P* value associated with the IPW treatment effect estimate. By block permutation, we mean that permutations are done within strata defined by the preprogram variables used in the randomization protocol: cohort, gender, number of siblings, mother’s IQ, and high-risk index. (vi) Multiple hypothesis stepdown *P* values associated with (v). The multiple hypothesis testing is applied to blocks of outcomes. Blocks of variables that are tested jointly using the stepdown algorithm are delineated by horizontal lines. *P* values ≤ 0.10 are in bold type. We use weight-for-length ≤ 85th percentile for being “at-risk overweight” under 24 months, and BMI-for-age ≥ 85th percentile for being overweight for 24 months and older (45). See table S14 for complete estimation results.

Variable	Control mean	Treatment mean	Difference in means	Conditional treatment effect	Block <i>P</i> value	Stepdown <i>P</i> value
<i>Health care at age 30</i>						
Health insurance coverage at age 30	0.857	0.760	–0.097	–0.159	0.943	0.943
Buys health insurance at age 30	0.357	0.400	0.043	–0.027	0.511	0.810
Hospital or doctor office care when sick at age 30	0.929	0.800	–0.129	–0.131	0.875	0.964
<i>Physical development in childhood</i>						
At risk overweight (CDC) at 3 months	0.192	0.190	0.002	–0.036	0.418	0.757
At risk overweight (CDC) at 6 months	0.423	0.167	0.256	0.212	0.040	0.237
At risk overweight (CDC) at 9 months	0.360	0.143	0.217	0.181	0.169	0.548
At risk overweight (CDC) at 12 months	0.478	0.208	0.270	0.141	0.055	0.276
At risk overweight (CDC) at 18 months	0.440	0.318	0.122	0.118	0.311	0.669
Overweight (CDC) at 24 months	0.412	0.174	0.238	0.195	0.143	0.517
Overweight (CDC) at 36 months	0.261	0.143	0.118	–0.020	0.202	0.556
Overweight (CDC) at 48 months	0.192	0.409	–0.217	–0.247	0.944	0.944
Overweight (CDC) at 60 months	0.261	0.273	–0.012	–0.050	0.554	0.781
Overweight (CDC) at 96 months	0.174	0.350	–0.176	–0.230	0.943	0.985
Weight-for-length change 0–24 months (CDC)	0.857	0.918	–0.062	–0.052	0.658	0.688
Weight-for-length change 0–24 months (WHO)	1.129	1.215	–0.085	–0.006	0.660	0.660

importance of these early growth patterns is shown in Fig. 2. Fig. 2, A and B, shows the evolution of BMI-for-age during childhood for males by treatment status. It is noticeable that, while the BMI-for-age of the treatment group is always centered around the median for the reference population, the control group experiences a surge in the first year, which peaks at 18 months, becomes partially attenuated, and then exhibits diverging growth patterns after 5 years of age. It is striking that, when we consider the early growth trajectory by obesity status in adulthood (Fig. 2, C to F), those who are obese or severely obese in their mid-30s are already on a trajectory of above-normal BMI in the first 5 years of their lives. The effects of the intervention on early physical development are less pronounced for females (lower panel of Table 4 and Figs. 3 and 4). Fig. 4A and Table 4 show that there are significant differences in mean BMI-for-age and in the prevalence of being overweight, respectively, in the first 2 years of the intervention. These differences, however, fade out by the end of the daycare treatment. As observed for males, the females who are severely obese in their mid-30s are already on a trajectory of higher BMI-for-age in the first years of their lives (Fig. 4, E and F).

Conclusions

This paper analyzes recently collected biomedical data for the ABC intervention. Children ran-

domly assigned to the treatment group for ages 0 to 5 have a significantly lower prevalence of risk factors for cardiovascular and metabolic diseases in their mid-30s. Treated males have a healthier body mass in their childhood years. These early benefits persist into adulthood. The precise mechanisms through which these effects are obtained remain to be determined. It may be improved health due to access to pediatric care and proper nutrition in the early years, improved noncognitive skills as in the Perry study (50), improved cognitive skills, or some combination of all three factors. The bundled nature of the treatment does not provide the necessary independent variation in the components of the intervention that would allow us to examine the sources of treatment effects. A simple mediation analysis (presented in tables S19 and S20) suggests that half of the effect of the treatment on hypertension and obesity in the mid-30s may be mediated by the BMI of the child around 1 year of age, while no statistically significant role seems to be played by the availability of health insurance or improved socioeconomic status at age 30. However, the estimated mediation effects are not precisely determined, so these findings are necessarily speculative. Whatever the channel, our evidence supports the importance of intervening in the first years of life and suggests that early childhood programs can make a sub-

stantial contribution to improving the health of adult Americans and reducing the burden of health care costs. An intervention that lasted 5 years and cost \$67,000 [in 2002 dollars (51)] produced sustained and substantial health benefits. Early childhood interventions are an unexplored and promising new avenue of health policy.

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44. The LMS method is an age- and gender-dependent transformation that translates BMI measures into z scores. The transformation is applied to two widely used population-based reference data: (i) the 2000 CDC growth standards; and (ii) the 2006 WHO child growth standards scale. The LMS method transforms the information on the median (M), coefficient of variation (S), and skewness (L) of the BMI distribution of these population-based reference data into a Box-Cox power function. This information is then used to transform BMI measures into z scores.
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49. A reduction in BMI can be caused by either better nutrition and/or more physical exercise. Physical environment is certainly important: the Frank Porter Graham (FPG) Child Development Institute included a large open space, and outside play was a part of the daily routine (11:30 am and 3:00 pm); however, this is likely to have played an important role only after the toddlers had started walking. Of course, better nutrition does not necessarily come only in the daycare center: The treated children could have enjoyed more nutritious food at home as well, both because their preferences for food might have been affected and because their parents were counseled by the pediatricians on site during the physical exams. Finally, (47) provides evidence—based on the What We Eat in America 2003–2004, combined with the National Health and Nutrition Examination Survey (NHANES) 2003–2004—that Head Start participants consume similar level of calories as non-Head Start participants during evenings and weekends but fewer calories in the morning and in the afternoon during the week.
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Supplementary Materials

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Materials and Methods
Supplementary Text
Tables S1 to S26
References (52–112)

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Structure of the Yeast Mitochondrial Large Ribosomal Subunit

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Mitochondria have specialized ribosomes that have diverged from their bacterial and cytoplasmic counterparts. We have solved the structure of the yeast mitoribosomal large subunit using single-particle cryo-electron microscopy. The resolution of 3.2 angstroms enabled a nearly complete atomic model to be built de novo and refined, including 39 proteins, 13 of which are unique to mitochondria, as well as expansion segments of mitoribosomal RNA. The structure reveals a new exit tunnel path and architecture, unique elements of the E site, and a putative membrane docking site.

Mitochondria are organelles in eukaryotic cells that play a major role in metabolism, especially the synthesis of adenosine triphosphate (ATP). During evolution, mitochondria have lost or transferred most of their genes to the nuclei, substantially reducing the size of their genome (1). In yeast, all but one of the few remaining protein-coding genes encode sub-

units of respiratory chain complexes, whose synthesis involves insertion into the inner mitochondrial membrane along with incorporation of prosthetic groups (2). For the translation of these genes, mitochondria maintain their own ribosomes (mitoribosomes) and translation system. The mitochondrial ribosomal RNA (rRNA) and several transfer RNAs (tRNAs) are encoded by the mitochondrial

genome, whereas all but one of its ribosomal proteins are nuclear-encoded and imported from the cytoplasm. Mitoribosomes have diverged greatly from their counterparts in the cytosol of bacterial and eukaryotic cells and also exhibit high variability depending on species (table S1) (3). Several genetic diseases map to mitoribosomes (4). In addition, the toxicity of many ribosomal antibiotics, in particular aminoglycosides, is thought to be due to their interaction with the mitoribosome (5).

Mitochondrial translation in the yeast *Saccharomyces cerevisiae* (6) has been used as a model to study human mitochondrial diseases (7). The 74S yeast mitoribosome has an overall molecular weight of 3 MD, some 30% greater than that of its bacterial counterpart. It consists of a 54S large subunit (1.9 MD) and a 37S small subunit (1.1 MD).

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High-resolution crystal structures have been solved for bacterial (8, 9) and eukaryotic ribosomes (10–12), as well as for an archaeal large subunit (13). In comparison, the only structures to date for mitoribosomes come from cryo-electron microscopy (cryo-EM) reconstructions at 12 to 14 Å (14–16).

The very low yields of mitoribosomes from most tissues have impeded their crystallization. However, the advent of high-speed direct electron detectors (which allow accounting for particle movement during imaging) (17, 18) and improved algorithms for the classification and alignment of particles (19) allowed us to determine the structure of the large subunit of the yeast mitoribosome (hereafter referred to as 54S) to an overall resolution of 3.2 Å, resulting in a nearly complete, stereochemically refined atomic model.

Structure Determination

Ribosomes purified from yeast mitochondria were contaminated with cytoplasmic ribosomes and free 54S (fig. S1A). The use of maximum likelihood image classification techniques (20) enabled sorting of these classes of particles into separate groups (fig. S1B). A preliminary reconstruction of the 74S mitochondrial ribosome yielded a density map with overall resolution of 3.6 Å. However, the small subunit was found to adopt a wide range of orientations with respect to the large subunit, resulting in markedly worse resolution for this region (~5 Å). Attempts to further classify the mitoribosomes into separate groups failed to yield maps that were suitable for de novo model building of the small subunit. We focused our subsequent efforts on the large subunit only. Density for this region was improved further by reconstructions that combined the large subunit regions of particles consisting of the 74S mitoribosome with those of the free 54S subunit, in a refinement in which the small subunit was masked away. The resulting total of 47,124 particles yielded an overall resolution of 3.2 Å by the “gold standard” Fourier shell correlation (FSC) = 0.143 criterion (fig. S2A) (21).

The local resolution (22) is better than 3 Å for most of the interior of the 54S subunit (Fig. 1 and fig. S2B) but decreases toward the periphery, especially for elements such as the L1 and L7/L12 stalks, which are often disordered even in crystal structures of the ribosome. Even at the surface, the resolution mostly appears to be better than 4 Å (fig. S2C).

The high resolution for much of the molecule allowed us to construct a nearly complete model for the large subunit, including one-third of the molecule for which no previous structural homology was known. In some regions, the quality of the map allowed identification of amino acids directly from side-chain densities without any additional information, leading to an unambiguous assignment of specific mitoribosomal proteins to their corresponding densities. For other regions, the identification of specific proteins required a combination of secondary structure prediction, homology modeling, and molecular replacement (23). X-ray crystallographic tools such as Coot

(24) and REFMAC (25) were modified for model building, refinement, and cross-validation (23). The final model (Fig. 2A) has good stereochemistry and agreement with the density (table S2).

The model does not include surface exposed regions for which only partial density is visible, such as the L1 and L7/L12 stalks. In addition, some of the mitochondria-specific rRNA expansions are highly mobile and thus could not be completely resolved. Therefore, we included only the parts for which clear density is apparent in appropriately sharpened maps. The relative orientation of the two subunits in the major class is shown in fig. S3.

Overall Structure

Both protein and RNA moieties are substantially larger than their bacterial counterparts. From the total mass of 1.9 MD, 0.4 MD are from mitochondria-specific proteins and extensions of known bacterial homologs, 0.2 MD are from rRNA expansion segments (ESs), and 1.3 MD have structural equivalents in bacteria. Most of these additional elements occur on the surface of the conserved core (Fig. 2B), giving the 54S a markedly different overall shape from the large subunits of cytoplasmic ribosomes.

In particular, there is an expansion of the central protuberance (CP) and a new protuberance formed at the membrane-facing surface. In both cases, the rRNA expansion serves as a scaffold for the addition of new proteins. Such progressive expansion shares principles with the development of modern ribosomes themselves from primordial ancestors (26). In addition to these changes at the surface, there is a substantial remodeling of the tunnel through which the nascent protein chain proceeds as it emerges from the ribosome.

rRNA Expansion Segments

The 21S RNA of the 54S subunit contains 3296 nucleotides, of which 2714 have been modeled in

our structure. A secondary structure diagram was constructed using base-pairing information directly from the structure, supplemented by predictions for the unmodeled regions (Fig. 3 and figs. S4 and S5). Conserved rRNA helices are numbered corresponding to those in *Escherichia coli* (27), with ESs denoted by the preceding conserved helix. Despite the lower sedimentation coefficient relative to *E. coli* 23S, there has been a considerable expansion of rRNA, resulting in 392 additional nucleotides spread over 11 ESs. Only a few insertions correlate with those of the eukaryotic ribosome. However, as in the eukaryotic case, many of the insertions are flexible expansion segments that project out from the core and are not well resolved in the map. It is not clear whether this similarity is a coincidental growth of complexity by neutral means (28) or whether both evolved independently under selective pressure for functional reasons.

Mitochondria-Specific Protein Elements

Of the 39 proteins built into the 54S, 26 share homology with bacterial ribosomes, 8 are present in both yeast and mammalian mitochondria, and 5 are unique to yeast mitochondria (table S3 and fig. S6). Following a recent proposal by the ribosome community, proteins universal to all domains of life have a “u” prefix and bacterial numbering; those with only a bacterial homolog have a “b” prefix and bacterial numbering; and proteins unique to mitochondria have an “m” prefix. Bacterial ribosomal proteins L18, L20, and L25 are not present in the yeast mitoribosome. However, a homolog of L35 that was previously thought not to be part of the mitoribosome (29) was clearly identified in the maps. Mitochondrial homologous recombination protein 1 (MHR1) was also found to be a component of the mitoribosome (29). Density is absent for mL53, mL54, and mL61, which were present in our sample as judged by

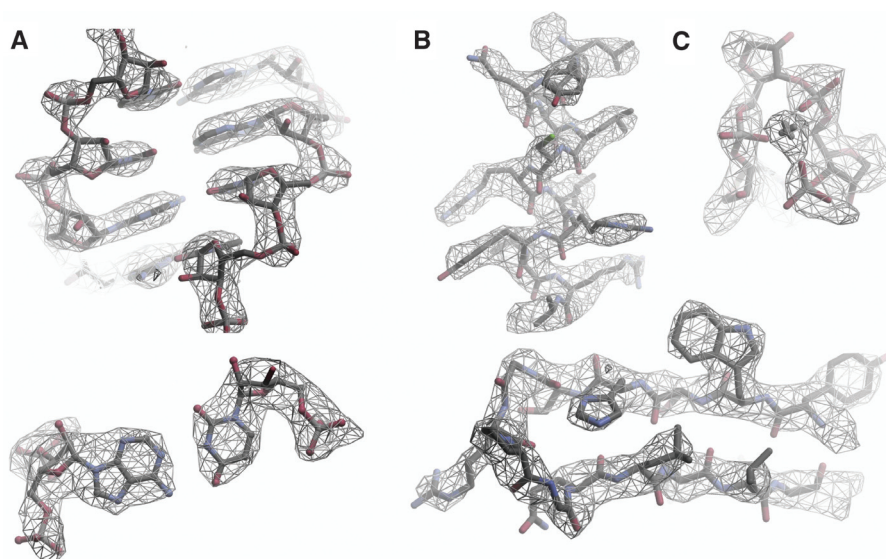


Fig. 1. Examples of the density in cryo-EM maps of the 54S subunit. (A) RNA showing a double helix (top) and a base pair (bottom). **(B)** An α helix and a β turn in proteins showing side chains. **(C)** Mg ion that coordinates a tight turn in RNA.

mass spectrometry, indicating that they are disordered or dissociated during grid preparation.

Most of the homologous proteins are considerably larger, with an average mass of ~25 kD compared to ~13.5 kD for their bacterial counterparts (table S3 and fig. S6). Similar to eukaryotic ribosomes, many of the extensions to bacterial proteins consist of long tails and loops extending from globular domains. For example, uL24 has approximately three times the number of residues and extends 115 Å across the surface of the mitoribosome (fig. S7A). As in the eukaryotic ribosome (11, 12), many of these extensions contact other extensions and unique proteins and rRNA (Fig. 2), suggesting a role in the assembly of mitoribosome-specific components. Some of the flexible extensions are highly basic and probably assist rRNA folding by neutralizing negative charge, as suggested for bacterial and archaeal ribosomes (13, 30). Only the extensions to uL17 and bL27 form compact domains. uL17 is characterized by a four-helix bundle fold, whereas bL27 forms a

domain with no homology to known structures (fig. S7B); both domains interact with rRNA.

As a result of protein extensions, the number of protein-protein contacts in the 54S is not only larger than in bacteria, but also exceeds that observed in the eukaryotic large subunit (11). Each protein in the mitoribosome has on average 4.5 neighbors (fig. S8), in comparison with only 1.5 in bacteria. Despite many more proteins overall, the number that contact only rRNA is halved relative to bacteria. Some of the new interactions include bridging different sites of the subunit (normally performed by 5S rRNA in cytoplasmic ribosomes), whereas others are involved in the architecture of the exit tunnel.

All of the 13 mitochondria-specific proteins interact with rRNA, including those that have domains not previously shown to have a role in binding nucleic acids. Of these, all except mL43 and mL49 are bound to rRNA ESs. Analyses of the unique proteins show that three of them have similarity with RNA binding proteins (mL44, mL49,

and mL57), two with DNA binding proteins (mL58 and mL60), two with nucleotide-binding proteins (mL38 and mL46), and two with lipid-binding proteins (mL38 and mL50; table S3). These proteins have lost their catalytic or ligand-binding residues and perform other roles in the mitoribosome.

The Central Protuberance (CP)

One site of major expansion is the CP, which because of its location is thought to facilitate communication between various functional sites of the ribosome in a manner that is not fully understood (31, 32). In bacterial, archaeal, and eukaryotic ribosomes, the CP consists of a separate 5S rRNA core interacting with several ribosomal proteins and 23S rRNA.

Yeast 54S lacks 5S rRNA and the 5S RNA-binding proteins L18 and L25. It is extensively remodeled (Fig. 4A) and consists of mitochondria-specific proteins (mL38, mL40, and mL46), RNA expansion clusters (82-ES and 84-ES1), and the extensions of the five homologous proteins (uL5, uL16, bL27, bL31, and bL33). These elements do not structurally mimic 5S rRNA but occupy its location in bacterial ribosomes (Fig. 4B). The bottom “toe” of 5S rRNA is replaced by 44-ES1, which interacts with uL16 and mL60. The large extension of bL27 binds the 82-ES1-3 to form part of the CP, illustrating how expansions in both proteins and rRNA have been used to reform the CP (fig. S7B). Despite the absence of 5S rRNA, the CP is triple the volume of the bacterial version. There is a larger network of interactions with the rest of the large subunit, as well as potential new contacts with the head of the small subunit (fig. S3).

A Membrane-Facing Protuberance

A second site enriched with mitochondria-specific proteins and rRNA is located adjacent to the mouth of the exit tunnel and thus must be close to the point of attachment of the ribosome to the translocon in the membrane (fig. S9). This protuberance consists of mitochondria-specific mL43, 44, 50, 57, and 58 (Fig. 2A) and rRNA expansion segments 0-ES1, 0-ES2, and 44-ES3. Two inactive members of the ribonuclease III family, mL44 and mL57, form a heterodimer and bend the 0-ES by an angle of 90°, forming an “L-shaped” helix (Fig. 5, B and C). The rest of the protuberance involves a second rRNA ES (44-ES3), the C-terminal part of mL50, N- and C-terminal extensions of uL22 and mL43, which binds predominantly to helix 46.

Yeast mitoribosomes have been shown to be permanently associated with the inner mitochondrial membrane in both translationally active and inactive states (33, 34). Moreover, mitoribosomes remain docked to the membrane even in the absence of the protein insertion machinery associated with the tunnel exit, indicating that additional and as yet uncharacterized anchors exist (35). The location of the membrane-facing protuberance makes it a candidate to be involved in the tethering of mitoribosomes to the membrane, independent of nascent peptide insertion.

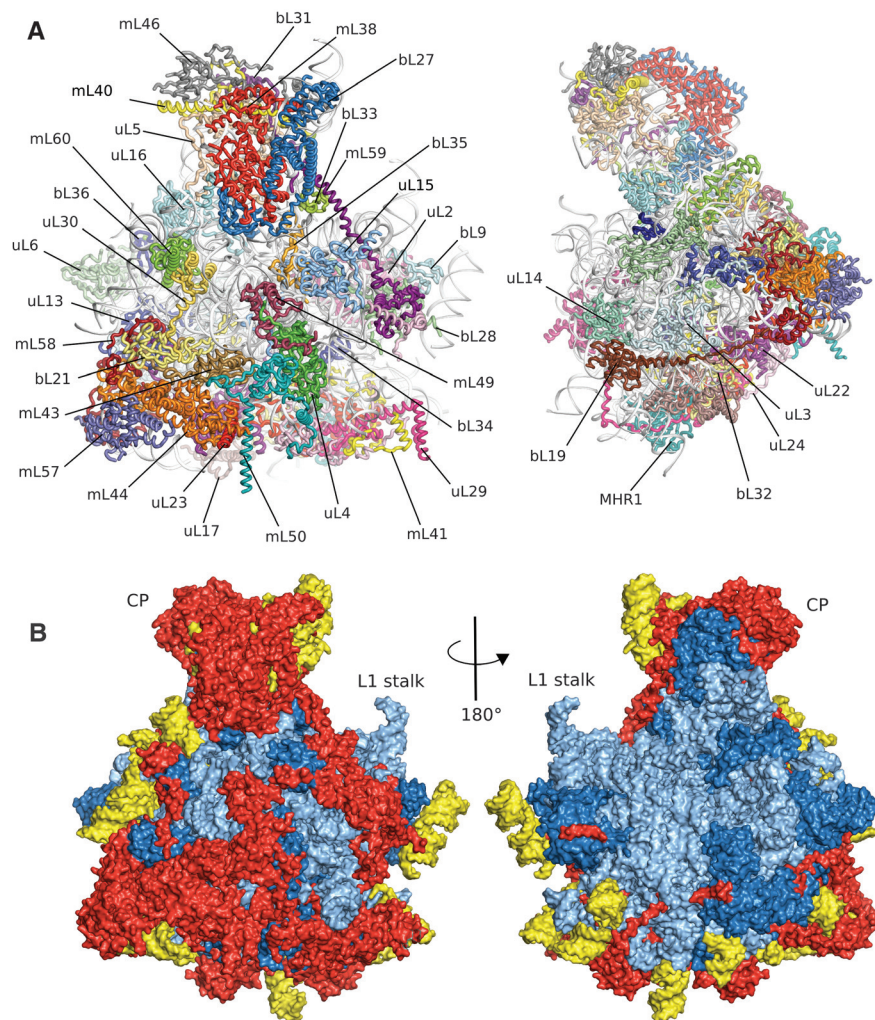


Fig. 2. Overview of the yeast 54S ribosomal subunit. (A) Cytoplasmic and side views of final model with 21S rRNA colored in gray. (B) Cytoplasmic and interface views colored showing regions conserved with bacteria (blue), found in both yeast and mammalian mitochondria (red) and present only in yeast mitochondria (yellow).

E-Site tRNA

The yeast mitoribosomes copurified with a tRNA bound in the exit (E) site. The density for the complete tRNA is relatively weak, presumably because of a combination of partial occupancy and mixed species of tRNA (fig. S10A). Nonetheless, we were able to model the acceptor stem including the conserved 3' CCA. The terminal adenine intercalates between bases 2688 (*E. coli*: 2421) and 2689 (2422) in a manner identical to bacteria and eukaryotes (fig. S10B). The tRNA appears to be stabilized by an additional contact not seen in bacteria. A C-terminal extension in bL33 creates a highly electropositive patch that stabilizes the phosphate backbone of the tRNA acceptor stem (fig. S10C). The extension of bL33 is stabilized by a change in the rRNA path prior to helix 82-ES1, which is itself partially stabilized by the unique protein mL59. Thus, the E site of ribosomes from archaea (36), bacteria, (9) and mitochondria (this work) all bind the terminal A76 in an essentially identical manner by intercalating between two purines of rRNA, while at the same time the rest of the E site has diverged among these classes of ribosomes.

The Exit Tunnel

During translation, the growing nascent chain proceeds from the peptidyl transferase center (PTC) through a tunnel to the opposite end of the large subunit where it emerges ~100 Å away. Given its universality, the tunnel is highly conserved among previously studied ribosomes. Despite this conservation, a superposition with bacterial ribosomes showed that the path of the bacterial tunnel is blocked in the 54S by a section of a long mitochondria-specific extension to uL23 that is well anchored in the 54S on either side and thus unlikely to be displaced (Fig. 6A). Instead, a new tunnel path is seen that deviates from the bacterial one ~60 Å from the PTC (Fig. 6B). This is the only channel in the yeast mitoribosome that extends from the PTC to the exterior of the ribosome. The boundaries of the new tunnel are defined by extensions of uL22, uL23, and uL24, and by rRNA 3-ES. The tunnel leads to a new exit site located roughly 35 Å away from where the bacterial tunnel would have emerged (Fig. 6, B and C, and fig. S9) and is surrounded by two additional unique proteins, mL44 and mL50, which are also involved in forming the membrane-facing protuberance (Fig. 6B). This suggests that the attachment to a translocon-like entity for insertion of the nascent peptide into the mitochondrial membrane must be very different in the 54S than in bacterial ribosomes. In support of this idea, uL24 was chemically found to form cross-links with uL23, mL44, and mL50 (37). The exit of the tunnel is also wider than in bacteria, possibly to allow cotranslational assembly of oxidative phosphorylation complexes (35, 38).

The entrance to the tunnel just beyond the PTC is noticeably narrower than in all previously determined structures of the large subunit (Fig. 6D). In part this is due to a base pair between U2877 and A1958 that appears unique to yeast

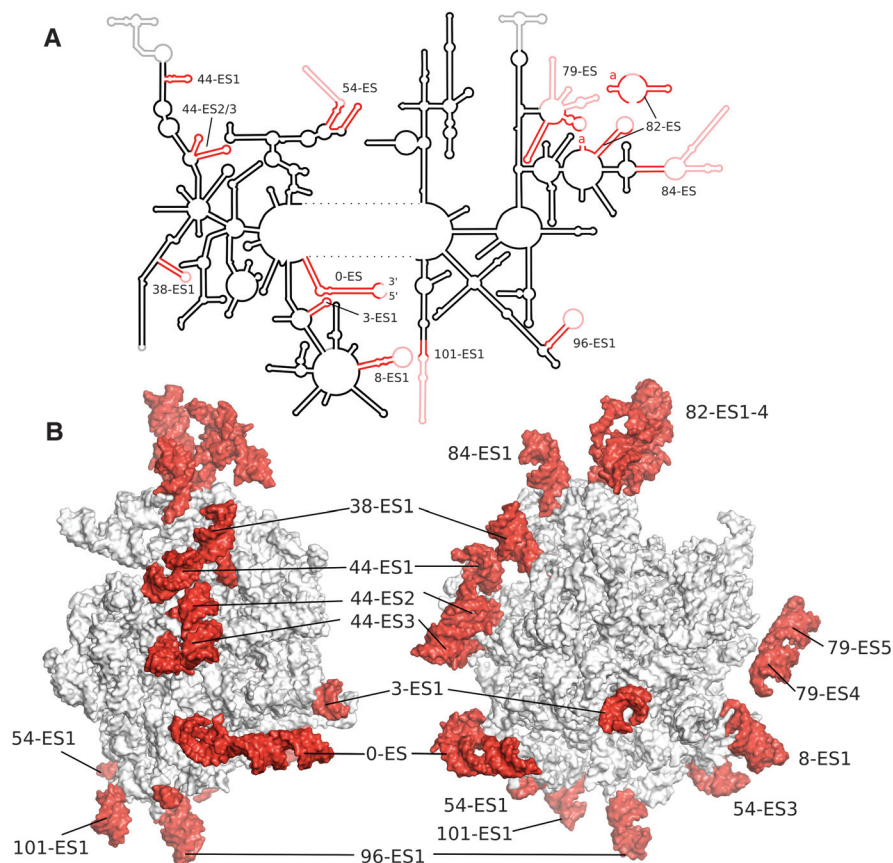


Fig. 3. RNA of the 54S subunit. (A) Schematic diagram of the secondary structure of 21S rRNA. (B) Two views of tertiary structure showing mitochondria-specific ESs in red labeled after the conserved helix according to standard numbering (see text).

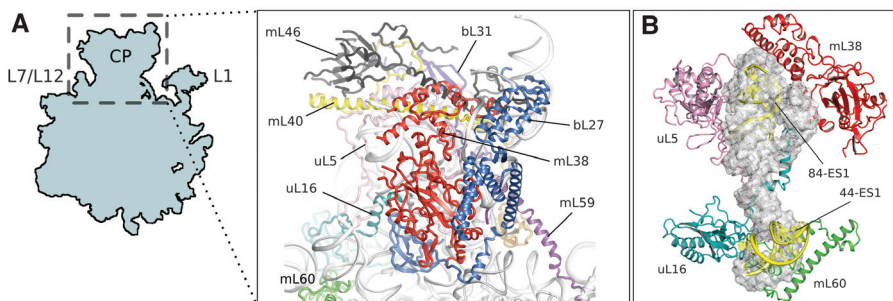


Fig. 4. Central protuberance (CP) of the 54S subunit. (A) Proteins involved in the CP. (B) Elements that replace 5S rRNA (gray) in the CP.

mitochondria and could only be identified on the basis of the structure. This pair brings opposing strands of 21S rRNA closer together by about 8 Å. This location is the binding site of macrolide antibiotics, which, unlike many aminoglycosides, do not bind to mitochondrial ribosomes (39) and thus do not have the same toxicity. A superposition of the macrolide erythromycin in its binding site shows that it would clash with the narrower tunnel entrance in mitochondria, so in yeast mitoribosomes there is an additional mechanism for resistance to these antibiotics beyond sequence variation of the binding pocket. Beyond the entrance, the tunnel is lined by conserved elements

(Fig. 6C), including the constriction site formed by uL22 and uL4. In addition, near the point where the path diverges from the path of the bacterial tunnel, two aromatic residues of uL22 and uL24 narrow the tunnel to a diameter of ~9 Å (Fig. 6C).

This work shows that recent advances in cryo-EM can be used to determine structures of comparable quality to x-ray crystallography but from much smaller amounts of more heterogeneous material. It also reveals that this highly divergent ribosomal subunit differs substantially from those of bacteria, eukaryotes, and archaea, with several unique features including an altered path of the exit tunnel.

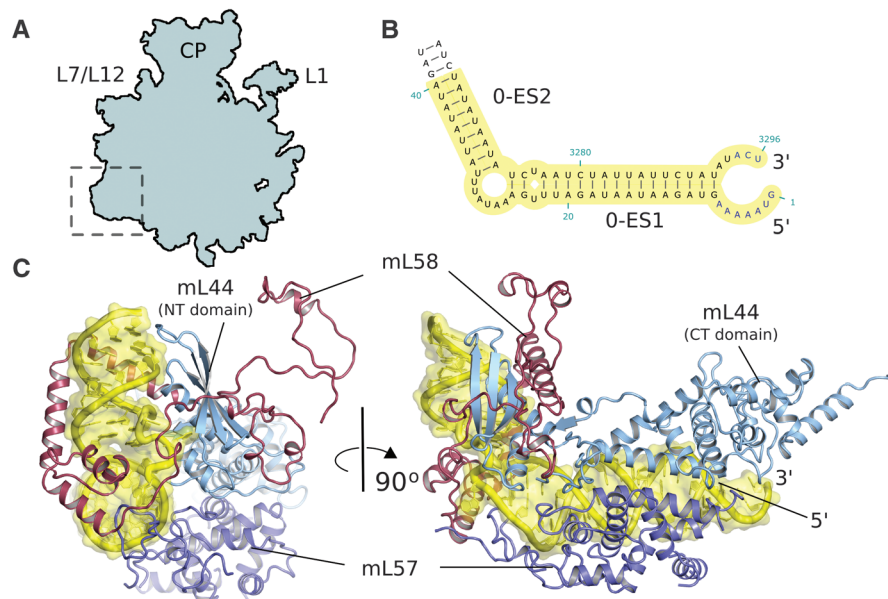


Fig. 5. A membrane-facing protuberance. (A) Site of expansion of the 54S relative to bacteria. (B) ESO of 21S rRNA serves as an anchor for the protuberance. (C) Two views of the protuberance showing how mitochondria-specific proteins bind to the expansion segment.

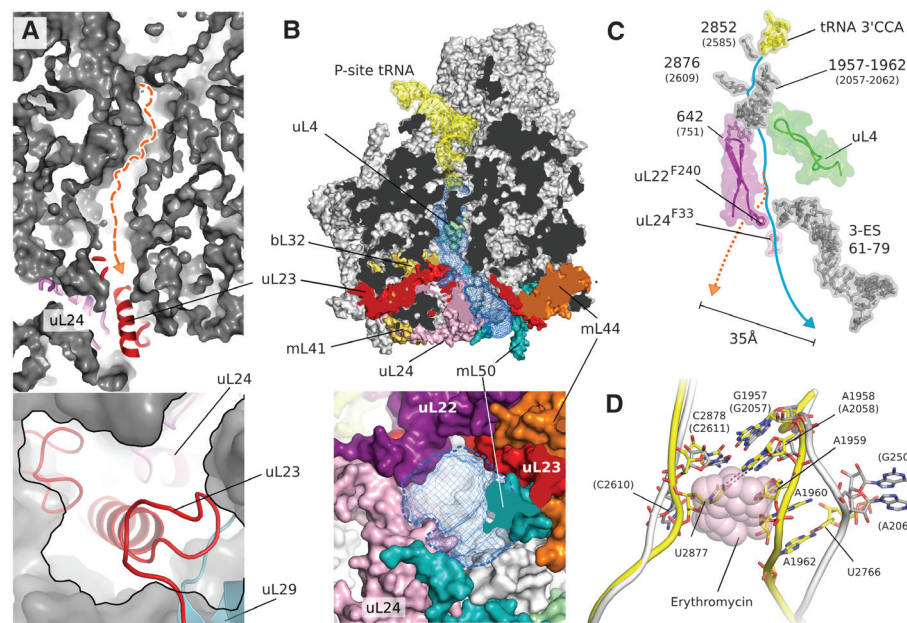


Fig. 6. The putative exit tunnel in the 54S subunit. (A) The bacterial tunnel (top, path in orange) is blocked by a mitochondria-specific extension to uL23 (detail in bottom panel). (B) The 54S tunnel (blue mesh) has been extensively remodeled by mitochondria-specific protein elements (colored) (top), resulting in a tunnel exit (bottom) that is substantially different from that in bacteria. In (A) and (B) the lower panels are 90° rotations of the upper panels. (C) Conservation of elements that make up the entrance and top part of the tunnel. The constriction at the top is narrower, and two aromatic amino acids that line the tunnel make a close approach. The path of the bacterial tunnel (orange) would exit 35 Å away. (D) Narrowing of the 54S tunnel entrance (yellow) due to an AU base pair not found in bacteria (white) that would cause a steric clash with macrolides such as erythromycin (pink).

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Supplementary Materials

www.sciencemag.org/content/343/6178/1485/suppl/DC1
Materials and Methods
Figs. S1 to S10
Tables S1 to S3
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Molecular Gas Clumps from the Destruction of Icy Bodies in the β Pictoris Debris Disk

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Many stars are surrounded by disks of dusty debris formed in the collisions of asteroids, comets, and dwarf planets, but is gas also released in such events? Observations at submillimeter wavelengths of the archetypal debris disk around β Pictoris show that 0.3% of a Moon mass of carbon monoxide orbits in its debris belt. The gas distribution is highly asymmetric, with 30% found in a single clump 85 astronomical units from the star, in a plane closely aligned with the orbit of the inner planet, β Pictoris b. This gas clump delineates a region of enhanced collisions, either from a mean motion resonance with an unseen giant planet or from the remnants of a collision of Mars-mass planets.

Debris disks are the end product of collisional cascades of kilometer-sized bodies orbiting stars (including the Sun) and are normally thought to contain negligible gas. At a distance of 19.44 pc (1) and age of 20 million years (2), β Pictoris (β Pic) is one of the closest, brightest, and youngest examples. Its edge-on disk was the first to be imaged in scattered light, showing the distribution of micrometer-sized dust grains (3). Various subsequent observations have provided evidence of infalling comets within a few astronomical units (AU) of the star (4), a massive planet at ~ 10 AU (5), as well as atomic gas extending out to ~ 300 AU (6); it is still unclear how these features are linked. By observing such debris disks at millimeter wavelengths, it is possible to trace the millimeter-sized dust and, by inference, the parent bodies of the collisional cascade, known as planetesimals (7).

We observed β Pic using the Atacama Large Millimeter/submillimeter Array (ALMA) at a

wavelength of 870 μ m in both the continuum and the 3–2 rotational line of ^{12}CO with a resolution of 12 AU (8). The continuum image (Fig. 1A) shows maxima in the surface brightness ~ 60 AU on either side of the star. At separations from 30 to 80 AU, the continuum is, on average, 15% brighter to the southwest. These results indicate that the millimeter-sized grains lie in a broad, slightly asymmetric belt nearly collocated with the disk of submicrometer-sized reflecting dust (9). The total flux of 60 ± 6 millijansky (mJy) corresponds to a dust mass of $4.7 \pm 0.5 \times 10^{23}$ kg [6.4 times the mass of the Moon (M_{Moon})], if we assume a standard dust mass opacity ($0.15 \text{ m}^2 \text{ kg}^{-1}$ at 850 μ m) and temperature of 85 K (10).

The planet β Pic b was close to maximum southwest elongation at the time of the observations, with a projected separation of 8.7 AU (11). Its location is coincident with a dip in the continuum surface brightness at a significance level of 4σ , suggesting that it may influence the innermost dust distribution.

ALMA also detected the disk in the ^{12}CO 3–2 transition (Fig. 1B), with a clear velocity gradient along the major axis, illustrated in the position-velocity (PV) diagram (Fig. 2). This shows the characteristic distribution of a broad belt of orbiting gas, with inner and outer radii of 50 and 160 AU, respectively, and a peak around 85 AU. No gas emission is seen inside 50 AU. The CO distribution is, on average, a factor of 2 brighter to the southwest, and a similar asymmetry was seen in the mid-infrared emission (12). Unlike the submillimeter continuum, the CO clump is offset by ~ 5 AU above the main disk plane (Fig. 1B) and is more closely aligned with the inner planet β Pic b and a secondary disk seen in scattered light (5, 9).

The CO 3–2 emission totals $7.6 \pm 0.8 \times 10^{-20} \text{ W/m}^2$. Assuming a range of possible excitation temperatures from 20 K [measured from ultraviolet (UV) absorption lines (13)] to 85 K [the dust equilibrium temperature (10)], this corresponds to a CO mass of 1.7×10^{20} kg

($0.0023 M_{\text{Moon}}$) within a factor of 2. Toward the star itself, the total CO column is $2.5_{(+2.5, -1.2)} \times 10^{15} \text{ cm}^{-2}$; results from UV absorption line measurements range from 0.6 to $2.1 \times 10^{15} \text{ cm}^{-2}$ (13), indicating that at least 10% of the CO along this line of sight lies in front of the star.

The attenuation through the disk due to dust or CO self-shielding is low, and, at radii >50 AU, CO is destroyed mostly by UV photons from the ambient interstellar medium (14). The CO photodissociation time scale in the unshielded outer disk will be ~ 120 years (15), which is substantially shorter than the 600-year orbital period at 85 AU. Unless we are observing β Pic at a very unusual time (that is, ≤ 100 years after a collision), the CO must be continuously replenished, with a steady-state production rate of $\sim 1.4 \times 10^{18} \text{ kg/year}$. The source of CO is likely the icy debris (grains, comets, and planetesimals) in the disk (16). In our own solar system, comets are mostly composed of refractory silicate grains together with H_2O and CO ices, with a typical $\text{CO}/\text{H}_2\text{O}$ ratio of 0.01 to 0.1 (17). Although CO sublimates at ~ 20 K, substantially lower than the 85-K dust temperature in the β Pic disk, it can be trapped in H_2O ice at temperatures up to ~ 140 K (18), which could occur in radiative equilibrium as close as 30 AU from β Pic (14). At larger distances, photodesorption or collisions can release this CO into the gas phase. Photodesorption (UV sputtering) of millimeter-sized ice grains (19) in a dust clump with 10% of the total disk mass would produce CO at a rate that is ~ 10 times lower than our observations show. The CO linewidth measured directly toward the star is $\sim 1 \text{ km/s}$ (Fig. 2), suggesting that the velocity dispersion is lower than the 6 km/s thought necessary for collisions to directly vaporize ice (20). However, icy parent bodies being shattered in multiple lower-velocity collisions may release much of the entrapped CO as gas, leaving the less volatile H_2O ice behind. To sustain the gas-production rate with a CO ice release fraction of 0.1, the required mass loss of colliding grains would be $\sim 1.4 \times 10^{19} \text{ kg/year}$, equivalent to the destruction of a large comet every 5 min. If sustained over the lifetime of β Pic, $\sim 50 M_{\text{Earth}}$ (where M_{Earth} is Earth's mass) of icy bodies will have been removed.

Continual replenishment of CO may explain another puzzle. In the absence of a braking mechanism, many of the atomic species (such as Na) found around β Pic should be blown out from the central star under the force of radiation pressure (21–23). Carbon is overabundant relative to other metals, by factors of 18 to 400 (21, 22), and has been proposed as the mechanism that allows the ionized and atomic gas to remain bound to the star. C^+ couples to other ions through Coulomb forces, and because C^+ feels weak radiation pressure from the central star, it tends to brake all the gas. The origin of the unusual carbon overabundance may be explained if CO is being rapidly released in the clump, then dissociated and ionized before being spread throughout the disk. In a steady state, the relative C^+/CO abundance

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implies that C^+ is being removed on a time scale of 3×10^3 to 10^4 years; because this is several orbital time scales at 85 AU, the braking gas can be spread throughout the disk.

If the gas is on circular Keplerian orbits, each point in the PV diagram corresponds to one of two points in the disk. Figure 3 shows possible deprojections of the data to form the face-on gas distribution (8). A compact clump of CO is found at a radius of 85 AU, which contains ~30% of the flux, along with an extended “tail” of emission. The radial distribution of the millimeter-sized

dust can also be derived (8, 12) and shows excess around this radius in the southwest (Fig. 3C). The millimeter-sized dust shows an abrupt drop in density around a radius of 130 AU, coincident with a change in slope in the radial distribution of scattered light. This was interpreted as a transition from a dust-producing planetesimal belt to a region dominated by submicrometer-sized grains blown out by radiation pressure (9, 24).

What is the origin of the CO clumps and corresponding mid-infrared and continuum features? There are two possible interpretations, both re-

quiring planet-mass objects in the outer disk, but with different predictions for the deprojected gas distribution. In the first (Fig. 3A), outward migration of a planet traps planetesimals into both the 2:1 and 3:2 mean motion resonances, resulting in a distribution with two clumps of asymmetric brightness on opposite sides of the star that orbit with the planet (25). Planetesimal collisions would occur most frequently in the clumps that would then be the most vigorous production sites of both CO and the short-lived micrometer-sized grains seen in mid-infrared images (12). The morphology

Fig. 1. Images of β Pic using ALMA in continuum and line emission. Both have been rotated by $+29^\circ$, the position angle of the main dust disk. The beam size (0.56×0.71 arc sec) is shown at the lower left, and the locations of the star and planet on the date of observations are indicated by the star and the cross, respectively. (A) Continuum emission at $870 \mu\text{m}$ from the millimeter-sized dust; the root mean square noise level is $61 \mu\text{Jy}$ per beam. (B) Total 3-2 CO line emission (rest frequency = 345.796 GHz); the noise level is $0.02 \times 10^{-20} \text{ W m}^{-2}$. The planes of the main and secondary dust disks (9) are shown by the dashed lines.

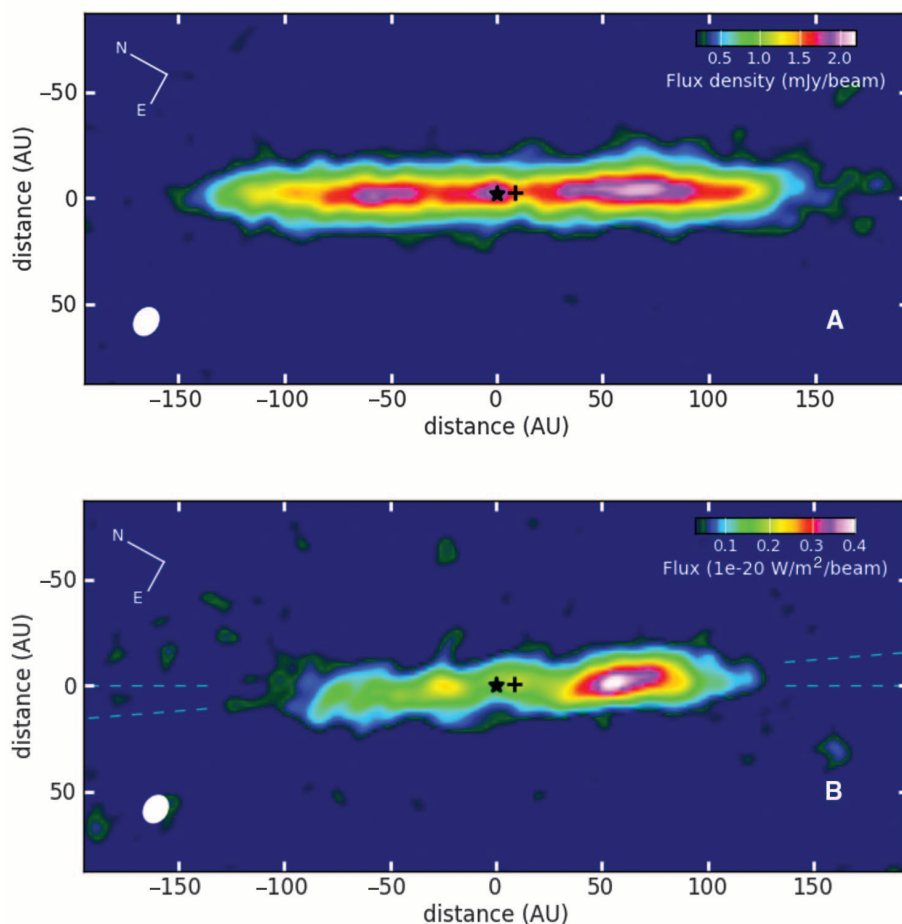
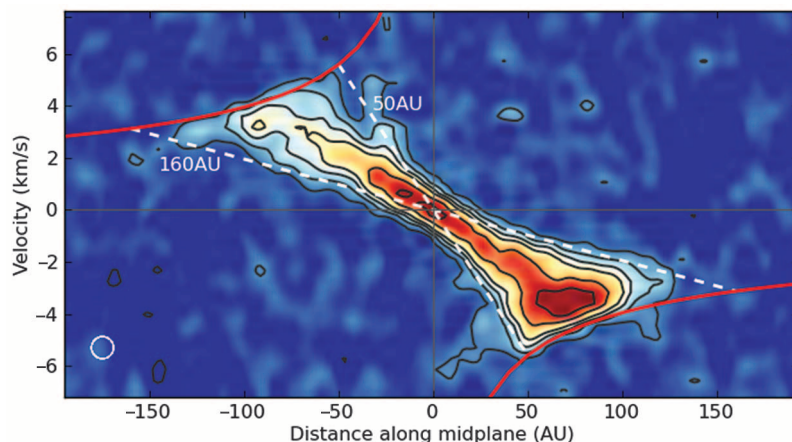
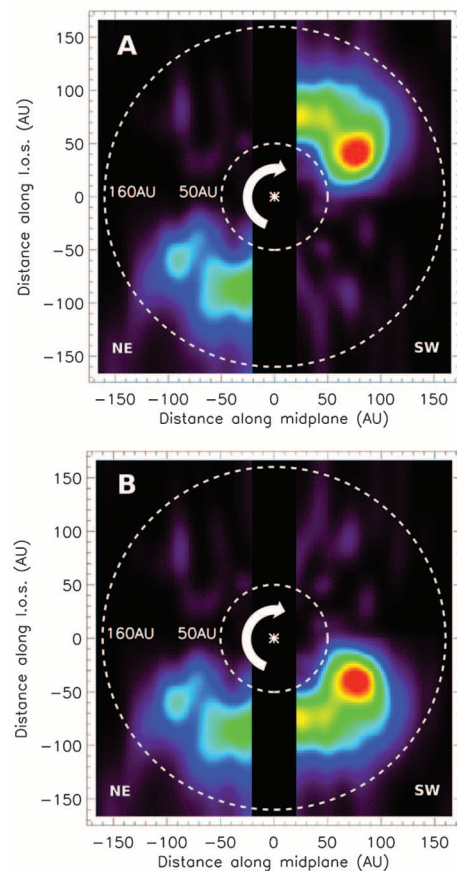


Fig. 2. Velocity distribution along the disk major axis. This image shows the observed velocity of the CO emission relative to the velocity measured toward the star (which has a centroid of $+20.3 \pm 0.1 \text{ km/s}$, in the barycentric reference frame). Gas on the brighter southwest side is approaching us; the two dashed white lines represent the velocity we would measure from gas at the inner and outer radii of the CO belt. The solid red lines show the true orbital velocity, the maximum observed velocity from gas at the tangential point, as a function of distance from the star, assuming a stellar mass of 1.75 times the mass of the Sun. Contours are 10, 20, 30, 40, 60, and 80% of the peak (0.06 Jy per beam). The spectral/spatial resolution is illustrated at the lower left.



of the clumps constrains the planet's mass and migration parameters (25). Notably, the planet must be $>10 M_{\text{Earth}}$ to have captured material into its 2:1 resonance, and it would currently be close to the inner edge of the gas-dust belt, $\sim 90^\circ$ in front of the southwest clump. The CO clump's tail would point in the opposite direction to the rotation, and its length is given by the ratio of CO photodissociation lifetime to the synodic period.

The alternative interpretation (Fig. 3B) is that the CO and micrometer-sized dust originate in a single recent collision (12, 26). Because the clump also contributes $\sim 10\%$ of the flux in our submillimeter continuum image, the parent body must have a mass approximately equal to that of Mars, as giant impacts typically release $\sim 10\%$ of the progenitor's mass as debris (27). Whereas collisional debris persists in a clump for only ~ 100 years after such an event, it is more likely that the collision occurred ~ 0.5 million years ago, with the asymmetry arising because the orbits of all collisional debris pass through the collision point, a region that would retain a high density and collision rate (26). Such a clump would be stationary, and the CO tail would lie in the rotation direction, with a length given by the ratio of photodissociation time to sidereal period (Fig. 3B).



Tentative evidence has been published for the clump's motion (28), which, if confirmed, would favor the resonance interpretation. Either way, these observations provide a valuable opportunity to ascertain the prevalence of planet-sized objects at large orbital distances in this disk.

The CO gas detected in β Pic is unusual among debris disks. So far, two others have been detected in CO [49 Ceti and HD21997 (16)] and one in O I [HD172555 (29)]. Most debris disks are presumed to contain icy grains, so we would expect all such systems to host CO and its photodissociation products. The high collision rates found in clumps may substantially enhance the gas abundance. If CO is formed in collisions, its brightness will scale as the collision rate, which is proportional to $v_r n_d^2$, where v_r is the collision velocity and n_d is the number of dust grains. Both n_d and v_r are enhanced in clumps due to resonances and giant impacts, increasing collision rates by factors of up to 10 to 100 (7, 26). The presence of these clumps can increase the overall collision rate and CO flux from a disk by one order of magnitude. The CO and compact clump in the β Pic disk indicate that this system is undergoing a period of intense activity driven by planets or planet collisions.

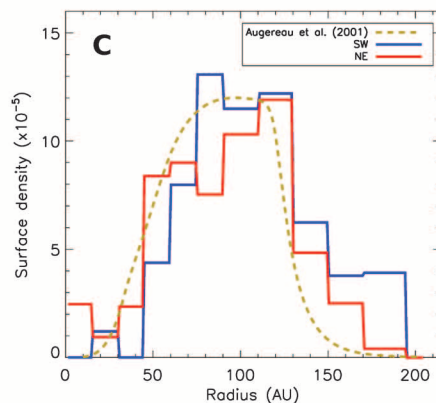


Fig. 3. Deprojected distributions of gas and dust around β Pic (8). (A and B) Two possible face-on distributions of the CO, assuming circular orbital motion. The dashed circles denote the inner and outer radii of the gas disk, the curved arrows indicate the direction of rotation, and the asterisks represent the location of the star. The distance along the line of sight (l.o.s.) is relative to the stellar position and is positive for gas on the far side of the star and is approaching us. (A) The two-clump distribution, interpreted as mean motion resonances with an inner planet. In this case, the bright southwest clump lies on the far side of the star and is approaching us. (B) Single-clump distribution, interpreted as a collision of objects with mass approximately equal to that of Mars. In this case, the southwest clump is stationary, and

the tail of CO points in the direction of rotation. (C) Radial distribution of millimeter-sized dust in the southwest (blue line) and northeast (red line) sectors of the disk, obtained by fitting consecutive annuli to the continuum distribution on the two sides (12). The dashed line is an axisymmetric model for the parent-body distribution, predicted from scattered light images of the submicrometer-sized grains (24). Surface density units are cross-sectional area per unit disk area, in AU^2/AU^2 .

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Observation of Brownian Motion in Liquids at Short Times: Instantaneous Velocity and Memory Loss

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Measurement of the instantaneous velocity of Brownian motion of suspended particles in liquid probes the microscopic foundations of statistical mechanics in soft condensed matter. However, instantaneous velocity has eluded experimental observation for more than a century since Einstein's prediction of the small length and time scales involved. We report shot-noise-limited, high-bandwidth measurements of Brownian motion of micrometer-sized beads suspended in water and acetone by an optical tweezer. We observe the hydrodynamic instantaneous velocity of Brownian motion in a liquid, which follows a modified energy equipartition theorem that accounts for the kinetic energy of the fluid displaced by the moving bead. We also observe an anticorrelated thermal force, which is conventionally assumed to be uncorrelated.

Einstein's seminal work on Brownian motion introduced a quantitative description of diffusion at long time scales (1). It led to experimental evidence of the molecular theory of matter and also explained why measurements of the velocity of Brownian particles were inconsistent with the equipartition theorem, a fundamental prediction of statistical mechanics (2, 3). Einstein considered the average velocity $\bar{v}_\tau$ measured over time interval τ . For diffusive Brownian motion, his theory predicted a root-mean-square value of $\bar{v}_\tau$ proportional to $\tau^{-1/2}$, diverging as τ approaches zero (4, 5). Einstein estimated that his simplification would break down at time scales on the order of τ_p , the momentum relaxation time. He claimed that the direct observation of the microscopic statistical mechanics of the Brownian particle, through measurement of the instantaneous velocity, would be "impossible" due to the demands on spatial and temporal resolution (4). A larger particle relaxes the demands on temporal resolution, but increases demands on position resolution owing to its slower thermal velocity. Recent work reported measurement of the instantaneous velocity of a Brownian particle in air, leading to confirmation of the equipartition theorem (5), providing access to dynamics otherwise concealed by thermal averaging (6), and allowing active suppression of thermal fluctuations (7). Velocity measurement in air was facilitated by advances in ultrasensitive detection and by the larger value of τ_p that results from the lower viscosity of air compared to liquid (5). In liquids, hydrodynamic coupling between the bead and the fluid dominates the dynamics of velocity fluctuations, whereas in air it is negligible. It is now understood that this coupling is important to both the fundamental statistics of Brownian fluctuations (8, 9) and to dynamics at time scales relevant to biological

systems (10), colloidal systems (11), and microfluidics (12).

Previous experiments reported observation of time-averaged hydrodynamic effects in Brownian motion for times as short as $\tau_f/15$, where τ_f is the time scale of bead-fluid interaction (13, 14). However, interesting effects of hydrodynamic interaction also extend to times that are orders of magnitude shorter than τ_f , while time-averaging imposes a thermal distribution of initial velocities and conceals the onset of equilibrium. In this work, we track the Brownian motion of dielectric microspheres (silica and barium titanate glass) suspended by an optical tweezer in water and acetone (Fig. 1). We achieve shot-noise-limited position sensitivity below $3 \text{ fm}/\sqrt{\text{Hz}}$ with a bandwidth of more than 50 MHz, which, combined

with an appropriate choice of system parameters, allows us to observe single-particle dynamics at times as short as $\tau_f/300$ and to resolve the Brownian particles' instantaneous velocity.

In Einstein's picture, mean-squared displacement (MSD) of Brownian trajectories is described by the diffusion equation, $\text{MSD}(\tau) \equiv \langle [\Delta x(\tau)]^2 \rangle = 2D\tau$ where D is the diffusion constant. Because of their fractal nature, purely diffusive trajectories have infinite length and thus an undefined instantaneous velocity, in contradiction of the equipartition theorem (5). This contradiction is resolved when the inertial mass of the particle is taken into account. Such a solution can be found by solving the stochastic Langevin equation, $m_p \dot{v} = F_{fr}(v) + F_{th}(t)$ where m_p is the mass of the particle, v is its velocity, $F_{fr}(v)$ is the damping force due to the fluid, and $F_{th}(t)$ is a random thermal force. The Langevin equation can be solved for the MSD and the corresponding velocity autocorrelation function $\text{VACF}(\tau) \equiv \langle v(t)v(t+\tau) \rangle = d^2/d\tau^2 (\text{MSD}(\tau)/2)$. The solution requires knowledge of the autocorrelation of the thermal force which, by the fluctuation dissipation theorem, can be determined from the dissipative component of F_{fr} (15).

The simplest model of ballistic Brownian motion assumes Stokes damping, $F_{fr} = -\gamma_s v$ with Stokes drag coefficient $\gamma_s = 6\pi\eta r$ for a spherical particle of radius r in a fluid with dynamic viscosity η . The resulting solution is characterized by an exponentially decaying VACF, with time-constant $\tau_p = m_p/\gamma_s$ (16). At long times, the model reduces to diffusive motion, whereas at short times, the MSD is proportional to τ^2 , and the VACF approaches its asymptotic value of $\langle v^2 \rangle = k_B T/m$, where k_B is Boltzmann's constant

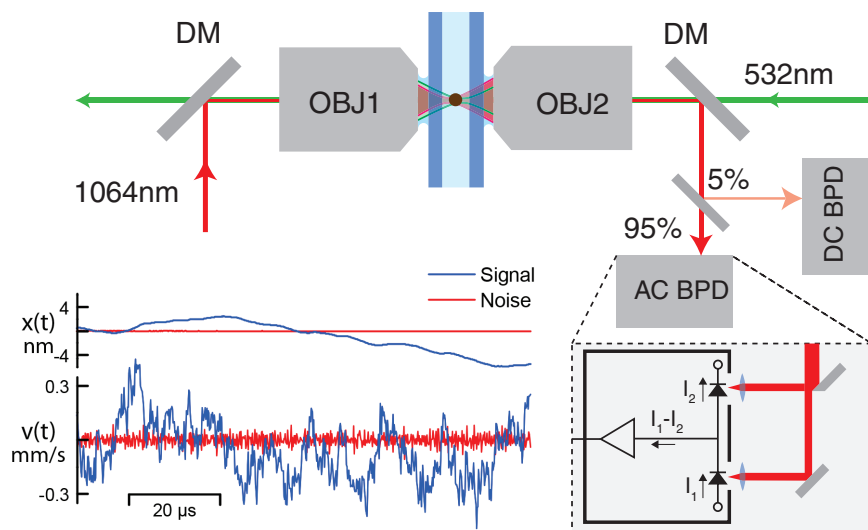


Fig. 1. A simplified schematic of the optical trap and position detection system. A microsphere is trapped by counterpropagating 1064- and 532-nm laser beams focused by microscope objectives (OBJ). The 1064-nm laser is then used to detect the horizontal motion of the bead, split between a low-power, dc balanced photodetector (DC BPD) and a high-power, ac-coupled balanced photodetector (AC BPD) (DM: dichroic mirror). Inset: A sample of the position (top trace) and velocity (bottom trace) of a trapped 3.7- μm -diameter barium titanate microsphere (blue line) recorded by the ac detector; the red line is the signal recorded with the same laser power but an empty trap.

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and T is the temperature, corresponding to the regime of ballistic motion. We refer to this solution as the Ornstein-Uhlenbeck model. Although Stokes damping holds for motion at constant velocity, two extra terms are required to correctly describe the force on a particle with nonzero acceleration.

In a dense fluid, the gravitational mass of an object is modified as a result of pressure from the fluid it displaces. Similarly, an object's inertial mass must be modified to account for the inertia of the displaced fluid. The effective particle mass is thus $m^* = m_p + m_a$, where, for a sphere in an unbounded fluid, the added (inertial) mass of the displaced fluid is $m_a = \frac{2}{3}\pi r^3 \rho_f$ (17), which is non-negligible when fluid density (ρ_f) is comparable to that of the particle (ρ_p). The equipartition prediction for the mean-squared thermal velocity of the hydrodynamically coupled bead is thus $\langle v_{th}^2 \rangle = k_B T / m^*$ for each Cartesian component of the velocity.

In a viscous fluid, memory of the acceleration of a sphere at one instant is retained by vorticity in the fluid flow. Vorticity is generated at the sphere's surface and gradually expands outwards, affecting the force on the sphere at later times. The difference between this dynamic force and steady-state Stokes damping is known as the Basset force (18, 19):

$$F_B(t) = -\gamma_s \sqrt{\frac{\tau_f}{\pi}} \int_{-\infty}^t \frac{\dot{v}(t')}{\sqrt{t-t'}} dt' \quad (1)$$

where $\tau_f = r^2 \rho_f / \eta$ is the time over which the vorticity expands a distance r , and $\dot{v}$ is the acceleration. The Basset force is not purely dissipative; energy from the entrained fluid can be returned to the particle. For short-time velocity fluctuations, the Basset force can dominate Stokes damping (fig. S3D), whereas for long-time fluctuations it can dominate inertial forces (fig. S3C).

At intermediate times it can dominate both. This behavior can be illustrated by considering the force required to accelerate an initially stationary sphere, $v(t < 0) = 0$ with constant acceleration at time $t = 0$, $\dot{v}(t \geq 0) = a_0$. The contribution by the Basset force grows as $t^{1/2}$, surpassing the inertial force $-m_a a_0$ of the added mass at $t \approx \tau_f/100$ and dominating the Stokes damping force $-\gamma_s a_0 t$ until $t \approx \tau_f$. Addition of the Basset force to the Langevin equation requires modification of the statistics of the random thermal force, substantially changes the behavior of the VACF (20, 21), and could potentially cause deviation from a Gaussian velocity distribution (9).

Figure 1 shows a simplified schematic of our experiment. An optical tweezer is created by two counterpropagating laser beams (1064 and 532 nm, both ~ 200 mW) focused by two identical water-immersion microscope objectives (numerical aperture 1.23). A flow-cell between the two objectives is used to introduce particles into the tweezer. The horizontal component of the trapped particle's displacement is measured with split-beam detection (5). The 1064-nm beam is split between two detectors, a high-power (~ 100 mW), low-noise ac-coupled detector (whose response is shown in fig. S1) and a low-power dc-coupled detector (22). The signals from each detector are digitized and stored on a computer. The particle diameter d , the trap constant K , and the detector calibration coefficient are determined by using a least-squares fit of the MSD of the recorded trajectories (Fig. 2A). All of the data used in this paper come from 0.35-s recorded trajectories of a barium titanate microsphere in acetone ($d = 3.72 \pm 0.06 \mu\text{m}$, $K = 3.2 \pm 0.2 \times 10^{-4}$ N/m, $\tau_f = 8.5 \mu\text{s}$) and a silica microsphere in water ($d = 2.86 \pm 0.03 \mu\text{m}$, $K = 1.6 \pm 0.3 \times 10^{-4}$ N/m, $\tau_f = 2.0 \mu\text{s}$).

The uncertainty of each fit parameter is determined from the variance in the results of independent MSD fits for 10 subtrajectories.

Our measured MSD and VACF demonstrate that we can observe dynamics deep into the ballistic regime. At short times, the MSD (Fig. 2A) has slope 2 (in a log-log plot), a signature of the ballistic regime of Brownian motion. The harmonic potential causes the MSD to plateau around $\tau_k \equiv \gamma_s / K$, before the purely diffusive regime is reached. The transition is more evident in the VACF (shown normalized by $\langle v_{th}^2 \rangle$ in Fig. 2B). At short times the VACF decays, to first order, as $1 - \sqrt{t/\tau_v}$, where $\tau_v = (\pi/4)(\tau_f^2/\tau_f)$ (22). This faster-than-exponential decay results from a two-fold action of the Basset force, which increases both the strength of the damping force and the magnitude of thermal force fluctuations at short time scales. The more familiar long-time tails (8, 23–25) appear at times longer than τ_f . The barium titanate microsphere in acetone has a much larger τ_v (11 μs) compared to the silica microsphere in water (0.57 μs), owing to the comparatively larger ρ_p of barium titanate and smaller ρ_f and η of acetone. The larger value of τ_v facilitates instantaneous-velocity measurement.

The useful bandwidth of instantaneous-velocity measurement is limited not directly by the detector bandwidth, but by the noise floor of the detector. The presence of shot noise in position measurement results in b^3 growth of the mean-square noise in velocity measurement, where b is the bandwidth chosen for measurement. Noise can be reduced by decreasing b , but if b is too low, the measured velocity will give a smaller apparent mean kinetic energy than the equipartition thermal energy $E_{th} \equiv k_B T/2$. The effect of low-pass filtering on the mean-square value of the resulting velocity can be estimated from the

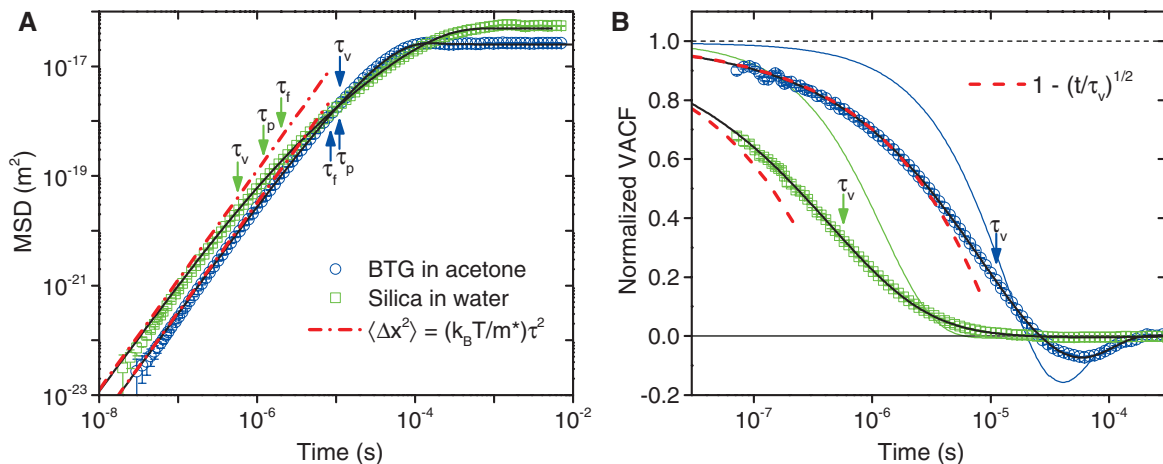


Fig. 2. Experimental and theoretical correlation functions from recorded trajectories of two different bead-fluid combinations. (A) Double-logarithmic plot of the MSD for an optically trapped barium titanate glass (BTG) bead (3.7 μm diameter) in acetone (blue circles; $\tau_p = 11.0 \mu\text{s}$, $\tau_f = 8.5 \mu\text{s}$, $\tau_v = 11.2 \mu\text{s}$), and a silica bead (2.8 μm in diameter) in water (green squares; $\tau_p = 1.2 \mu\text{s}$, $\tau_f = 2.01 \mu\text{s}$, $\tau_v = 0.57 \mu\text{s}$). The red dashed lines indicate the MSD of a particle moving at constant velocity. (B) Semilogarithmic plot of

the corresponding VACF normalized by $\langle v_{th}^2 \rangle \equiv k_B T / m^*$, (0.18 mm/s) 2 and (0.35 mm/s) 2 for the barium titanate and silica microspheres, respectively. The horizontal dashed black line guides the eye to the asymptotic value of the VACF at short times. The solid blue and green lines correspond to the Ornstein-Uhlenbeck model (which neglects hydrodynamic interactions). The dashed red lines correspond to the first-order approximation $1 - \sqrt{t/\tau_v}$. The solid black lines correspond to the full hydrodynamic theory (26).

cumulative velocity spectrum (shown normalized by $\langle v_{\text{th}}^{2*} \rangle$ in Fig. 3B), determined by integrating the velocity power spectral density from $\omega = 0$ to $\omega = 2\pi b$. For the filtered velocity to closely approximate the true velocity, b must be high enough to capture most of the area of the velocity power spectrum (Fig. 3A). Because the hydrodynamic velocity spectrum falls off less steeply at high frequency ($\omega^{-3/2}$) than the Ornstein-Uhlenbeck prediction (ω^{-2}), an accurate measurement of

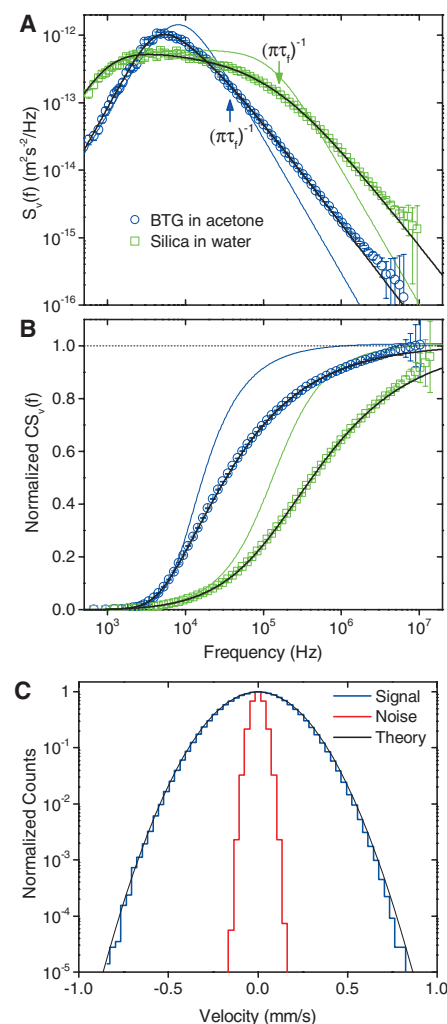


Fig. 3. Analysis of velocity measurements determined from observed Brownian trajectories. (A) Double-logarithmic plot of the velocity power spectral density (PSD) and (B) semilogarithmic plot of the normalized cumulative velocity PSD for the same particles as in Fig. 2. The solid blue and green lines correspond to the Ornstein-Uhlenbeck model, and the solid black lines correspond to the full hydrodynamic theory (26). The dashed black line guides the eye to the asymptotic approach toward unity. (C) The instantaneous-velocity distribution for the barium titanate microsphere in acetone (blue line) and the distribution of the velocity noise (red), both calculated with an averaging time of 0.16 μs . The black line shows the theoretical Maxwell Boltzmann distribution for a temperature of 291 K.

$\langle v_{\text{th}}^{2*} \rangle$ requires much higher b than expected from the Ornstein-Uhlenbeck model (22).

Even though the heavier barium titanate bead has lower $\langle v_{\text{th}}^{2*} \rangle$, its larger τ_v enables measurement further into the ballistic regime than for the silica microsphere in water. We achieve a noise floor of 2.9 fm/ $\sqrt{\text{Hz}}$ in the position spectrum for the silica microsphere in water and 2.1 fm/ $\sqrt{\text{Hz}}$ for the barium titanate microsphere in acetone (22), as reflected in fig. S2. Although the noise levels are similar, τ_v is too short to accurately measure the instantaneous velocity of the silica microsphere in water; the noise obscures a substantial fraction of the velocity spectrum. However, for the barium titanate microsphere in acetone, an averaging time of 0.16 μs per velocity sample allows resolution of the instantaneous velocity with a signal-to-noise ratio of ~ 14 dB. Figure 3C shows the resulting velocity distribution ($v_{\text{rms}} = 0.174$ mm/s), as well as that of the velocity noise ($v_{\text{rms}} = 34$ $\mu\text{m/s}$) measured without a bead in the trap but with the same detection power. The velocity distribution is within the experimental uncertainty of the predicted Maxwell-Boltzmann distribution ($v_{\text{rms}} = 0.180$ mm/s). The histograms were calculated from 2 million velocity points. The bin size in the velocity histogram was chosen to match the root-mean-square magnitude of the noise. Our measured distribution

corresponds to a mean kinetic energy $0.93 E_{\text{th}}$, to which the noise contributes $0.035 E_{\text{th}}$. A shorter averaging time would increase the fraction of kinetic energy observed at the cost of a lower signal-to-noise ratio.

Although the Ornstein-Uhlenbeck model predicts a thermal force with a white (single-sided) power spectral density of $S_{F_{\text{th}}} = 4k_B T \gamma_S$ (16), addition of the Basset term results in a colored component: $S_{F_{\text{th}}} = 4k_B T \gamma_S (1 + \sqrt{\omega \tau_f/2})$ (26). We use the mechanical response corresponding to our fit parameters to infer $F_{\text{th}}(t)$ from our recorded trajectories. A log-log plot of $S_{F_{\text{th}}}$ with its constant term subtracted reveals the $\sqrt{\omega}$ dependence of the colored component (Fig. 4A). Color in $S_{F_{\text{th}}}$ necessarily implies a nondelta autocorrelation function, which we also can observe (Fig. 4B). Interestingly, the force is anticorrelated, displaying a $-3/2$ power-law dependence over our measurement range. Although Stokes damping leads to a delta-correlated thermal force, consisting of uncorrelated “kicks,” hydrodynamic coupling effectively adds a negative tail to each kick, which is represented by an additional term $-\gamma_S k_B T \sqrt{\tau_f/4\pi\tau^{-3/2}}$ in the force autocorrelation.

Measurement of the cross-correlation between the particle’s velocity and the nondeterministic, thermal force $C_{vF_{\text{th}}}(\tau) \equiv \langle v(t)F_{\text{th}}(t+\tau) \rangle$ reveals the causal interplay between the particle

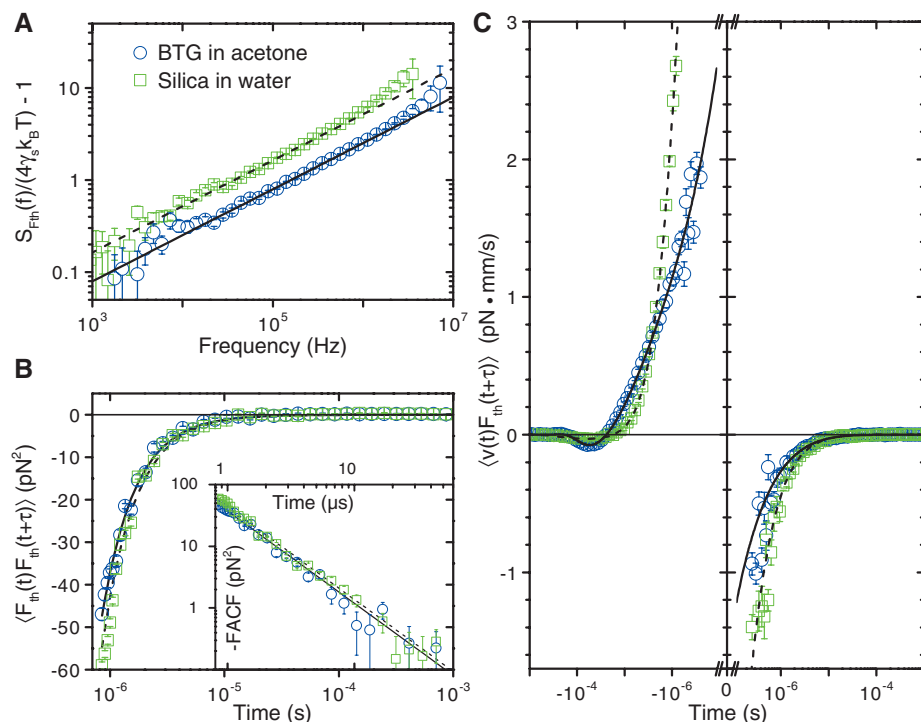


Fig. 4. Statistical properties of thermal force inferred from recorded Brownian trajectories. (A) Double-logarithmic plot of the colored component of the thermal force power spectral density for the same particles as in Figs. 2 and 3. (B) A semilogarithmic plot of the anticorrelations in the thermal force for the two beads. The inset contains a double-logarithmic plot of the absolute value of the force autocorrelation, showing its power-law dependence. (C) Semilogarithmic plot of the cross correlation of the thermal force with the particle velocity. The cross correlation function is asymmetric in time. In all three plots, the black solid and dashed lines correspond to the prediction of the full hydrodynamic theory for the barium titanate and silica microspheres, respectively (26, 27).

and the fluid (Fig. 4C). Unlike autocorrelation, which by definition is time-symmetric, $C_{vF_{th}}(\tau)$ is different for positive and negative τ . The existence of nonzero correlation between the velocity and the thermal force exerted on the bead at future times may seem counterintuitive. This too is a consequence of the hydrodynamic interactions. In the Ornstein-Uhlenbeck model it is exactly zero for $\tau > 0$; the velocity contains no information about future thermal force. Nonzero correlation for positive τ does not violate causality, however. The thermal force of the future is correlated to the thermal force of the past (Fig. 4B), and the velocity is correlated to the thermal force of the past, resulting in nonzero correlation between velocity and the future force (27).

Pushing the limits of detection has allowed us to resolve the trajectory in velocity space of Brownian particles in liquid, which, in typical experiments, is normally obscured by noise or concealed by averaging. Our measurements confirm a Maxwell-Boltzmann probability distribution for the velocity with the particle mass replaced by an effective mass that accounts for the inertia of the displaced liquid. Our observations agree with the predicted effects of hydrodynamic interactions on Brownian dynamics, including a faster-than-exponential decay of the

VACF and correlations in time of the random thermal force. Our techniques will find broad applications in the study of non-Newtonian fluids (28), effects of hydrodynamic interactions in confined geometries (29), and nonequilibrium statistical mechanics (30, 31).

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Supplementary Materials

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Materials and Methods

Supplementary Text

Figs. S1 to S3

References (32–35)

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Quantum Plasmon Resonances Controlled by Molecular Tunnel Junctions

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Quantum tunneling between two plasmonic resonators links nonlinear quantum optics with terahertz nanoelectronics. We describe the direct observation of and control over quantum plasmon resonances at length scales in the range 0.4 to 1.3 nanometers across molecular tunnel junctions made of two plasmonic resonators bridged by self-assembled monolayers (SAMs). The tunnel barrier width and height are controlled by the properties of the molecules. Using electron energy-loss spectroscopy, we directly observe a plasmon mode, the tunneling charge transfer plasmon, whose frequency (ranging from 140 to 245 terahertz) is dependent on the molecules bridging the gaps.

Quantum mechanical effects in plasmonic structures are believed to become important when two plasmonic resonators are placed so closely that electrons can tunnel across the gap (1–11). Direct experimental access to the resulting tunneling charge transfer plasmon (tCTP) mode is expected to open up new opportunities in, for instance, nanoscale optoelectronics, single-molecule sensing, and nonlinear optics (1). Experimental and theoretical studies so far have concluded that quantum mechanical effects are important only at length scales below 0.3 to 0.5 nm, close to the bond length of gold and silver (8–11). Such structures are technologically inaccessible; therefore, it is important to demonstrate the tCTP mode across gaps larger than a nanometer that can be fabricated

by state-of-the-art fabrication techniques (10). Unlike past works that investigated tunneling through a vacuum (12), we placed molecules in the gap because tunneling rates across molecules depend on the molecular structure and are much higher than across a vacuum. This approach made it possible to directly observe and control tCTPs experimentally in tunneling gaps up to at least 1.3 nm, depending on the type of molecules bridging the gap, and to move quantum plasmonics into the size domain that is accessible via bottom-up or top-down fabrication methods (10).

Quantum effects have been observed only indirectly as shifts in the bonding dipolar resonance plasmon mode (1, 9, 11). Our aim was to perform an experiment in which the presence of a tun-

neling barrier can be directly imaged while the tCTP mode is simultaneously measured spectroscopically by introducing two experimental innovations: The cross-sectional area of the tunnel junction was increased from a few nm² to roughly 10³ nm², and the tunneling rate across the nanogaps was increased by tunneling through molecules rather than vacuum.

Cuboidal silver nanoparticles were used (13), separated by SAMs with thicknesses of 0.5 to 0.6 nm forming metal-SAM-metal junctions through self-assembly (Fig. 1). The facets of the nanoparticles are atomically flat, which results in a very large cross-sectional area of around 10³ nm², maximizing the number of tunneling events across the junctions. The silver nanoparticles were functionalized with either saturated, aliphatic 1,2-ethanedithiolates (EDT) or aromatic 1,4-benzenedithiolates (BDT) (14). The lengths of EDT and BDT are similar, but they have very different HOMO (highest occupied

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molecular orbital)–LUMO (lowest unoccupied molecular orbital) gaps of 8 and 5 eV, respectively (15–17). Therefore, the tunneling rates across junctions with BDT molecules are higher than those junctions with EDT. The interaction between the two nanoparticles was optimized to avoid aggregation or misalignment by diluting the dithiols with 1-propanethiol (PT) (14). After self-assembly of the dimeric structures, they were deposited on a 30-nm-thick, electron-transparent silicon nitride membrane.

A simplified form of the Simmons equation (Eq. 1) is commonly used to approximate molecular tunnel junctions (18, 19)

$$J = J_0 e^{-\beta d}$$

with

$$\beta = 2\sqrt{\frac{2m\phi}{\hbar^2}} \quad (1)$$

where β ($\text{\AA}^{-1}$) is the tunneling decay coefficient, d (nm) is the width of the tunneling barrier, and the pre-exponential factor J_0 (A/cm^2) is the hypothetical current when $d = 0$; m is the mass of the charge carrier (kg), and $\hbar$ is the reduced Planck's constant. The value of β depends on the barrier height ϕ (eV). Tunneling rates through molecular bonds, so-called through-bond tunneling, are much higher ($\beta \approx 0.8$ to $0.9 \text{ \AA}^{-1}$ for saturated molecules and 0.1 to $0.3 \text{ \AA}^{-1}$ for unsaturated molecules) than through vacuum ($\beta = 2.9 \text{ \AA}^{-1}$) (20). Figure 1C shows the energy level diagram of the metal-SAM-metal junctions schematically. As indicated in Fig. 1C, in molecular electronics d is defined by the length of the molecule d_l (nm), and ϕ by the offset between the Fermi levels of the metal and the energy level of the molecular frontier orbitals. In contrast, when tunneling through

a vacuum is the dominant mechanism of charge transport, the barrier height equals the work function of the electrode materials and d equals the gap, d_g (nm), between the two electrodes—that is, the distance between the nanoparticles (21).

Instead of bringing the dimer particles in contact with electrical probes that will perturb the plasmon resonances, we used in our experiment a focused beam of energetic electrons in a scanning transmission electron microscope (STEM) as a contactless nanoprobe to excite and analyze the surface plasmon resonances in individual dimers. We positioned the electron probe opposite the gap at the long axis of a silver particle dimer, as illustrated in Fig. 1A, to excite plasmon modes—similar to lateral plane wave illumination of the dimer (14). During the plasmon excitation, the tunnel junction was therefore not exposed to the electron beam to minimize irradiation damage (14). The excitation of plasmon resonances results in an energy transfer from the electron beam to the particle system, which we analyzed with monochromated electron energy-loss spectroscopy (EELS) (22–24). The junctions were imaged before and after acquiring the EELS spectra to ensure that none of these junctions formed conductive metal filaments during the experiment (14). Thus, in all of our experiments, we could discriminate between the tunneling and conduction through metal filaments—CTP modes conclusively.

Figure 2A shows atomic-resolution TEM images of a silver nanoparticle dimer. The first peak in the histograms of values of d_g estimated from TEM images on a series of dimers is centered at 0.55 ± 0.08 nm for EDT and 0.67 ± 0.12 nm for BDT, which are close to d_l as expected for Ag-SAM-Ag structures. The second peak in these histograms is attributed to dimers with SAMs on both nanoparticles (Ag-SAM//SAM-Ag structures,

where “//” indicates a noncovalent contact). Inter-calating SAMs or incompletely removed polymer that was used in the nanoparticle synthesis may result in smaller and larger gap sizes than expected from the molecular lengths, as indicated schematically in Fig. 3.

Figure 2B shows EELS spectra recorded from junctions with SAMs of EDT and BDT. Three main plasmon peaks were observed around 2.2 eV, 3.2 eV, and 3.6 eV, which we assigned to the bonding dipolar plasmon mode (II), the transverse corner mode (III), and the transverse edge mode (IV), respectively, in agreement with the finite-element-model (FEM) simulations (Fig. 2D) (14, 21). A new low-energy plasmon mode is observed at 0.60 ± 0.04 eV for EDT and 1.01 ± 0.01 eV for BDT.

We assigned this plasmon mode to the tCTP based on our calculations that show the transfer of net charge between the cuboids (Fig. 2D, mode I). The plasmon resonances of the Ag-SAM-Ag system was simulated using a quantum-corrected FEM optical simulation model (14, 21). Briefly, the optical properties of the junctions are calculated through a quantum mechanical approach and then used to simulate the plasmon resonances of the Ag-SAM-Ag system. The model predicts that the tCTP mode strongly depends on ϕ , d , and gap field, E_{gap} (V/m). The value of ϕ is modeled analytically as $\phi = \alpha E_{\text{HL}}$, where $0 < \alpha < 1$ relates to the energy-level alignment, and E_{HL} is the HOMO-LUMO gap, which was obtained from single-molecule experiments (15–17). We assume $E_{\text{gap}} = 7 \times 10^8$ V/m throughout the calculations based on previously reported work (14, 21).

The nature of the charge transport—through-space or through-bond tunneling—was determined from EELS measurements on 32 junctions, for which the energy of the tCTP mode was plotted as a function of d_g (Fig. 2C). This graph shows that the tCTP depends only weakly on the value of d_g . The dotted lines in Fig. 2C show simulations of the tCTP energy shifts if through-space tunneling dominated, with $d = d_g$ (14). The solid lines are simulations for through-bond tunneling with $d = d_l$, where the through-bond tunneling distance depends on the length of the molecule and can be different from d_g when the molecules are not perfectly aligned in the gap. Figure 2C shows that through-bond tunneling has a much weaker dependence on gap size than through-space tunneling. The good agreement with the experimental results indicates that coherent through-bond tunneling is the dominant mechanism of charge transport (14).

Through-bond tunneling allows us to explore the tCTP mode across a large gap because the tunneling is less dependent on the gap size. EELS spectra were recorded on Ag-SAM//SAM-Ag junctions to study whether the tCTP mode could be observed over larger length scales up to 1.3 nm. Figure 3 shows a tCTP mode for the structures with BDT at 0.975 to 1.015 eV. The tCTP peak energy only weakly depends on d_g , which confirms

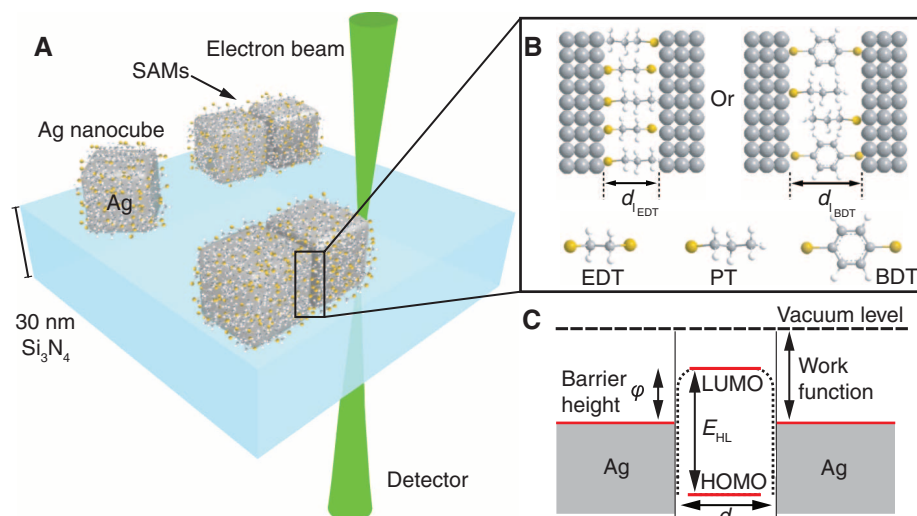


Fig. 1. Quantum plasmonic tunnel junctions. (A) Schematic illustration of the molecular tunnel junctions made of two silver nanoparticles bridged by a SAM on an electron-transparent silicon nitride membrane. The contactless electron nanoprobe was placed near the functionalized silver nanoparticles to excite and measure the surface plasmons of individual dimers. (B) The distance between two adjacent nanoparticles is determined by the thickness of the SAMs of EDT or BDT. (C) A schematic energy-level diagram of the junctions.

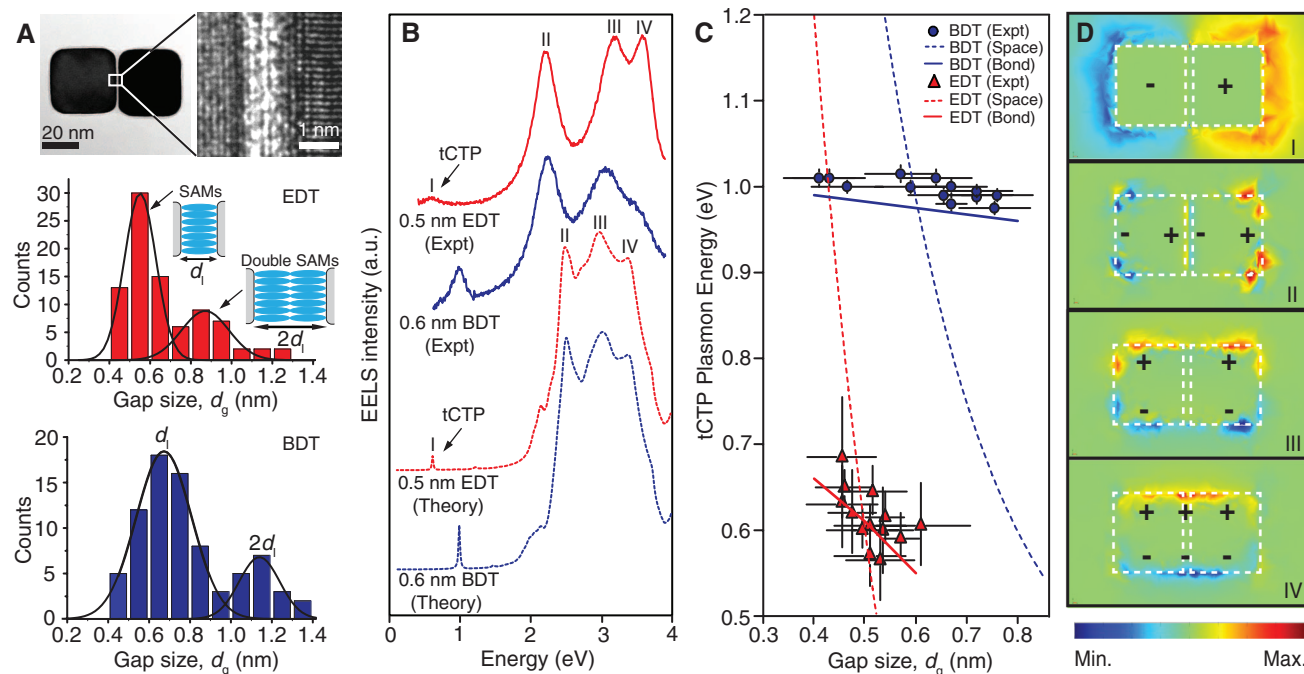


Fig. 2. Direct observation of quantum tunneling between plasmon resonators. (A) A high-resolution TEM image of the junctions and histograms of the gap-sizes (14). (B) Two examples of measured EELS spectra with the occurrence of quantum tunneling directly observed by the tCTP peak and quantum-corrected simulations of the extinction spectra, confirming the identification of the peaks. (C) Experimentally measured plasmon energy

as a function of gap size for dimers functionalized with monolayers of BDT (blue circles) and EDT (red triangles). Theoretical calculations for through-space and through-bond tunneling are shown as dotted lines and solid lines, respectively, for the two SAMs. (D) Simulated maps of the electrical-field distributions for the plasmon modes I to IV, corresponding with the spectral peaks.

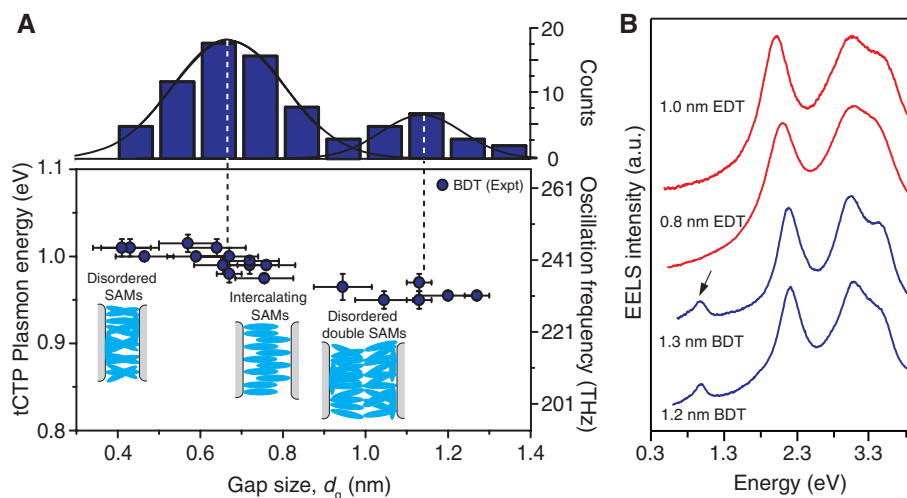


Fig. 3. Quantum plasmon resonances as a function of tunneling distance. (A) Experimentally measured plasmon energy as a function of gap size for BDT- (blue circles) functionalized dimers. The gap size varies between individual dimers because of structural disorder in the SAMs. (B) Measured EELS spectra for double SAMs of EDT (red) and BDT (blue). Tunneling was observed for the double-layer BDT but not in the double-layer EDT junctions.

that though-bond tunneling is the dominant mechanism of charge transport. The marginal difference in energy of the tCTP mode for single and double SAMs is likely due to strong π - π coupling between the BDT SAMs (25). For Ag-SAM/SAM-Ag structures with EDT, the tCTP mode was not observed because of the low β value, and because no π - π coupling occurs between aliphatic molecules, we expected its peak—if any—to be at

very low energies below the detection limit of our instrument.

By combining atomic-resolution imaging, single-particle spectroscopy, and monolayer molecular control, we have demonstrated quantum-mechanical electron tunneling at optical frequencies between plasmon resonators. By varying the self-assembled molecular monolayers in the junctions, we found that the plasmon-induced tunneling fre-

quencies could be controlled from 1.01 ± 0.01 eV, or 244 ± 3 THz, for a monolayer of BDT molecules, to 0.60 ± 0.04 eV, or 145 ± 10 THz, for a monolayer of EDT molecules. The mechanism of charge transport was coherent through-bond tunneling, which is only weakly dependent on the gap size. The relatively large distance of up to 1.3 nm over which the tunneling takes place in Ag-BDT/BDT-Ag junctions may provide potential for molecular control over quantum plasmonic systems through longer molecules to perhaps 4 to 5 nm (26)—that is, gap sizes that are currently accessible by top-down fabrication techniques. Our results show that tunneling can reconcile molecular electronics with plasmonics, opening up a whole new interdisciplinary field of exploration.

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Optical Broadband Angular Selectivity

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Light selection based purely on the angle of propagation is a long-standing scientific challenge. In angularly selective systems, however, the transmission of light usually also depends on the light frequency. We tailored the overlap of the band gaps of multiple one-dimensional photonic crystals, each with a different periodicity, in such a way as to preserve the characteristic Brewster modes across a broadband spectrum. We provide theory as well as an experimental realization with an all-visible spectrum, p-polarized angularly selective material system. Our method enables transparency throughout the visible spectrum at one angle—the generalized Brewster angle—and reflection at every other viewing angle.

The ability to control light has long been a major scientific and technological goal. In electromagnetic theory, a monochromatic electromagnetic plane wave is characterized (apart from its phase and amplitude) by three fundamental properties: its frequency, its polarization, and its propagation direction. The ability to select light according to each of these separate properties would be an essential step in achieving control over light (Fig. 1).

Tremendous progress has been made toward both frequency selectivity and polarization selectivity. Frequency selectivity (Fig. 1A) can be obtained, for example, by taking advantage of photonic band gaps in photonic crystals (1–5). Polarization selectivity (Fig. 1B) is accomplished, for example, by means of a “wire grid” polarizer (6) or by exploiting birefringent materials (7, 8). Methods based on interference and resonance effects have been explored for angular selectivity, but they have limited applications because they are sensitive to frequency.

An angularly selective material system should ideally work over a broadband spectrum. Such a system could potentially play a crucial role in many applications, such as high-efficiency solar energy conversion (9, 10), privacy protection (11), and detectors with high signal-to-noise ratios. Some progress has been made toward achieving broadband angular selectivity by means of metallic extraordinary transmission (12, 13), anisotropic metamaterials (14), combined use of polarizers and birefringent films (11), or geometrical optics

at micrometer scale (15). The first two of these mechanisms are difficult to realize in the optical regime; the other two work only as angularly selective absorbers.

Here, we introduce a basic principle to achieve optical broadband angular selectivity. Our result rests on (i) the fact that polarized light transmits without any reflection at the Brewster angle, (ii) the existence in photonic crystals of band gaps that prevent light propagation for given frequency ranges, and (iii) the band gap–broadening effect of heterostructures. First, we prove our fundamental idea theoretically for a single polarization and oblique incident angles, and also for both polarizations and normal angle of incidence. Second, we experimentally demonstrate the concept in the case of all-visible spectrum, p-polarized light. The demonstrator is transparent for all colors at one viewing angle and highly reflecting at every other viewing angle.

We begin by considering a simple quarter-wave stack with periodicity a , relative permeability μ of $\mu_1 = \mu_2 = 1$, and relative permittivities ϵ of ϵ_1 and ϵ_2 . In such a system, monochromatic plane waves with frequency ω propagate only in certain directions; propagation in other directions is not allowed because of destructive interference (3). Another way to look at this is through the photonic band diagram shown in Fig. 2A: Modes that are allowed to propagate (so-called extended modes) exist in the shaded region; no modes are allowed to propagate in the white regions (known as band gaps). In the photonic band diagram, modes with propagation direction forming an angle θ_i with respect to the z axis in Fig. 2 (in the layers with dielectric constant ϵ_i) lie on a straight line represented by $\omega = k_y c / (\sqrt{\epsilon_i} \sin \theta_i)$, where k_y is the y component (as defined in Fig. 2) of the wave vector $\mathbf{k}$ and c is the speed of light;

for general propagation angle θ_i , this line will extend through the regions of the extended modes as well as through the band gap regions.

However, for p-polarized light, there is a special propagation angle, known as the Brewster angle θ_B , for which the extended modes exist regardless of ω (dashed line in Fig. 2A) (8, 16):

$$\theta_B = \tan^{-1} \sqrt{\frac{\epsilon_2}{\epsilon_1}} \quad (1)$$

where θ_B is the Brewster angle in the layers with dielectric constant ϵ_1 . At θ_B , p-polarized light is fully transmitted for all frequencies at both interfaces (from ϵ_1 to ϵ_2 layers and from ϵ_2 to ϵ_1 layers). This condition is not sufficient to achieve angular selectivity; we also need to remove all the extended modes in other propagation directions. Because the location of the band gap scales proportionally to the periodicity of the quarter-wave stack, the effective band gap can be enlarged when we stack quarter-wave stacks with various periodicities together (17–19). The details of this process are illustrated in fig. S1 (20). As a proof of principle, in Fig. 2D we plot the band diagram of an ideal structure with $\epsilon_1 = 1$ and $\epsilon_2 = 2$ and the number of quarter-wave stacks approaching infinity. By doing this with a finite system of 50 stacks (10 bilayers in each stack), we can achieve an angularly selective range of less than 2° and a frequency bandwidth of $\geq 54\%$, similar to the size of the visible spectrum (Fig. 2G).

For s-polarized light, as there is no Brewster angle, this construction behaves as a dielectric mirror that reflects over a wide frequency range and over all incident angles (fig. S2) (20).

The mechanism above provides both angular selectivity and polarization selectivity, and is therefore useful in many applications. For example, in most optically pumped lasers, the pumping light comes in with a specific polarization and at one specific angle. A cavity built with both angularly selective and polarization-selective mirrors will allow the pumping light to get through, while at the same time trapping all the light with other propagation directions and polarizations inside the cavity.

The restriction on the polarization can be lifted by releasing the conventional requirement that $\mu_1 = \mu_2 = 1$. During the past decade, it has been demonstrated that metamaterials have the potential to achieve $\epsilon = \mu \neq 1$ in a broad frequency range (21–23). Consider two media with $\epsilon_1 = \mu_1 \neq \epsilon_2 = \mu_2$;

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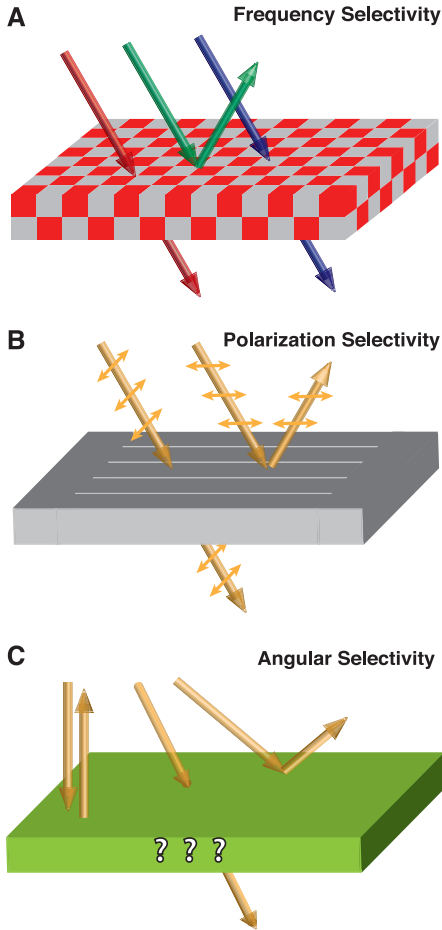


Fig. 1. Illustration of light selection on the basis of its fundamental properties. (A) Frequency selectivity provides control over transmission or reflection of different frequencies. Photonic crystals (such as omnidirectional mirrors) can select light in specific frequency bandwidths. (B) Polarization selectivity provides control over the transmission or reflection of different polarizations. An ideal polarizer selects light with a specific polarization. (C) Angular selectivity provides control over the transmission or reflection of incident angles; so far, achieving broadband selectivity has remained elusive.

under those circumstances, there is no reflection at the interface at normal incidence because the two media are impedance-matched, where the impedance Z is defined as $Z = \sqrt{\mu/\epsilon}$. The off-axis reflectivity can be calculated directly from the generalized Fresnel equations (8):

$$\left(\frac{E_r}{E_i}\right)_\perp = \frac{\frac{1}{Z_i} \cos \theta_i - \frac{1}{Z_t} \cos \theta_t}{\frac{1}{Z_i} \cos \theta_i + \frac{1}{Z_t} \cos \theta_t} \quad (2)$$

and

$$\left(\frac{E_r}{E_i}\right)_\parallel = \frac{\frac{1}{Z_i} \cos \theta_i - \frac{1}{Z_t} \cos \theta_t}{\frac{1}{Z_i} \cos \theta_i + \frac{1}{Z_t} \cos \theta_t} \quad (3)$$

where the subscripts i and r denote incident light and reflected light, respectively, and the subscripts $\perp$ and $\parallel$ indicate the direction of the electric field

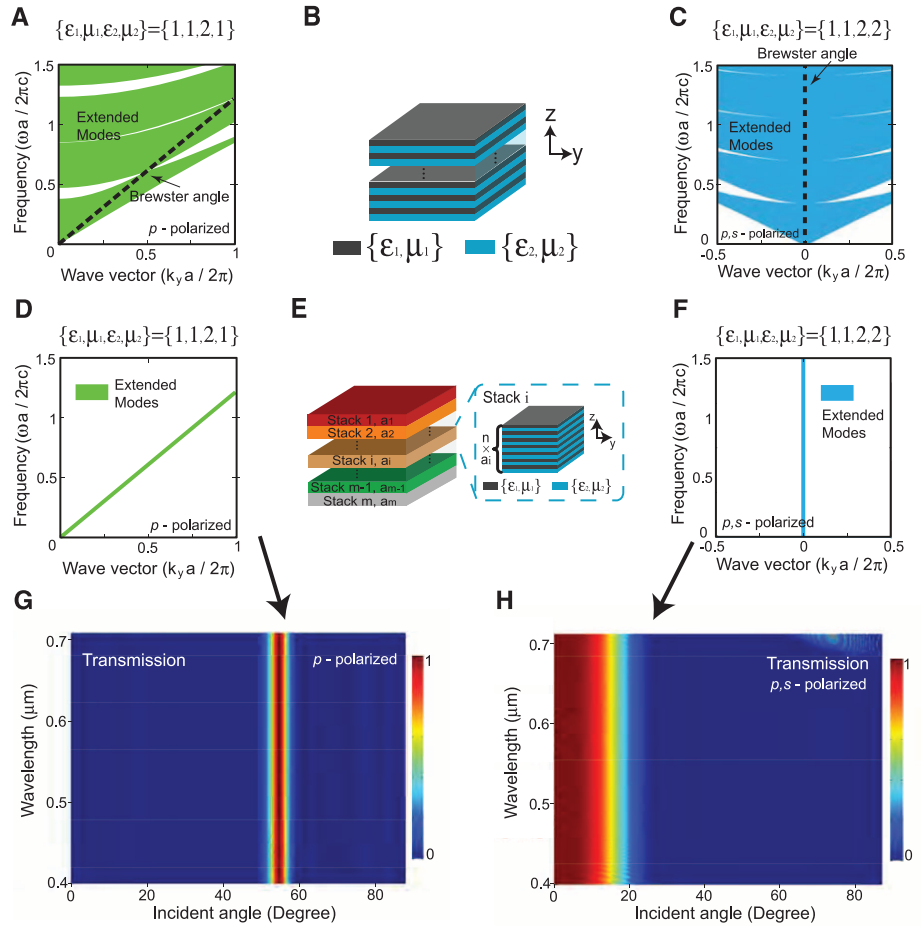


Fig. 2. Theoretical illustration. (A) Extended modes (shaded regions) for off-axis propagation vectors $(0, k_y, k_z)$ in a quarter-wave stack with two materials having $\epsilon_1 = 1$ and $\epsilon_2 = 2$, respectively. The green region indicates modes with $\mathbf{E}$ fields polarized in the yz incidence plane (p -polarized). The dashed black line corresponds to the Brewster angle θ_B in both layers. (B) Schematic layout of a simple quarter-wave stack. (C) The same plot as in (A), but with $\epsilon_1 = \mu_1 = 1$, $\epsilon_2 = \mu_2 = 2$, and for both p - and s -polarizations. (D and F) Extended modes for an ideal heterostructure with $(m, n) \rightarrow \infty$. (E) Schematic layout of the heterostructure stacking mechanism. (G) P -polarized transmission spectrum of 50 quarter-wave stacks at various periodicities. Each quarter-wave stack consists of 10 bilayers of $\{\epsilon_1 = 1, \epsilon_2 = 2\}$ materials. The periodicities of these quarter-wave stacks form a geometric series $a_i = a_0 r^{i-1}$ with $a_0 = 200$ nm and $r = 1.0212$, where a_i is the periodicity of i th stack. See (20) for a more detailed discussion of this stacking process. (H) P - and s -polarized transmission spectrum for a structure that has the same number of stacks and layers per stack as in (G), but with $a_0 = 140$ nm and $r = 1.0164$, and with different material properties: $\epsilon_1 = \mu_1 = 1$, $\epsilon_2 = \mu_2 = 2$.

$\mathbf{E}$ with respect to the plane of incidence. When $Z_i = Z_t$, the reflectivities for s - and p -polarized light become identical. In particular, the Brewster angle is the same for both polarizations ($\theta_B = \theta_i = \theta_t = 0^\circ$). As a proof of principle, we plot the band diagram of a quarter-wave stack with $\epsilon_1 = \mu_1 = 1$ and $\epsilon_2 = \mu_2 = 2$ in Fig. 2C. As in the previous case, we can broaden the band gaps by stacking quarter-wave stacks with various periodicities together (Fig. 2F); this approach gives rise to ultra-broadband angular selectivity at normal incidence for both polarizations (Fig. 2H).

Other than using photonic band gaps to remove unwanted extended modes, there might exist even more optimized ways to forbid light from propagating in unwanted directions: The narrowness of angular selectivity can be optimized using numerical tools (17, 24) to further enhance

the performance of the material system. Examples of optimizations based on three different physical mechanisms are shown in fig. S3 (20).

To show the feasibility of the method described above, we present an experimental realization for the $\epsilon_1 \neq \epsilon_2$, $\mu_1 = \mu_2 = 1$ case. The sample was fabricated with the bias target deposition (BTD) technique (25) at 4Wave Inc. using SiO_2 ($\epsilon_1 \approx 2.18$, $\mu_1 = 1$) and Ta_2O_5 ($\epsilon_2 \approx 4.33$, $\mu_2 = 1$) on a $2 \text{ cm} \times 4 \text{ cm}$ fused silica wafer (University Wafer Inc.). The sample consists of 84 layers in total (Fig. 2E). There are six bilayer stacks ($m = 6$), each bilayer stack consisting of seven bilayers ($n = 7$), with the thicknesses of each layer equal in a given stack. The periodicities of the six bilayer stacks form a geometric series with $a_i = a_0 r^{i-1}$ for the i th stack, where $a_0 = 140$ nm and $r = 1.165$. For index-matching purposes, the whole sample

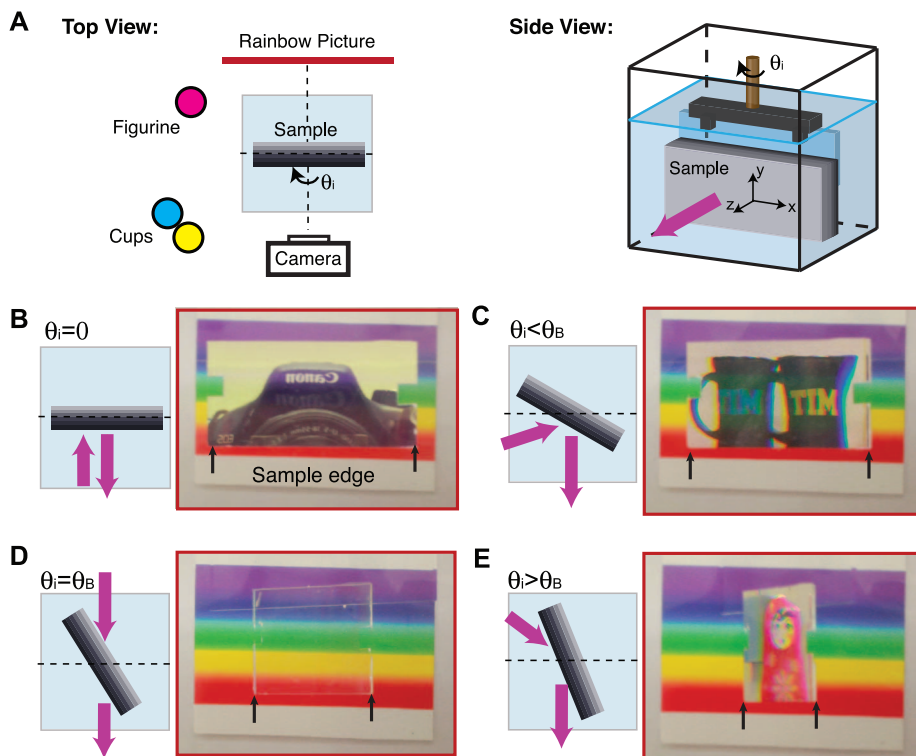


Fig. 3. Experimental setup and observation. (A) Schematic layout of the experimental setup. The system is immersed in a liquid that is index-matched to $\epsilon_1 = \epsilon_{\text{SiO}_2} = 2.18$. (B) Normal incident angle setup. The sample behaves as a mirror and reflects the image of the camera. (C) $\theta_i = 30^\circ$ setup. The sample behaves as a mirror and reflects the image of MIT cups in the lab. (D) $\theta_i = \theta_B = 55^\circ$ setup. The sample becomes transparent for the entire visible regime for p-polarized light. (E) $\theta_i = 70^\circ$ setup. The sample behaves as a mirror and reflects the figurine placed at the corner of the table. In (B) to (E), a polarizer is installed on the camera so that it detects only p-polarized light.

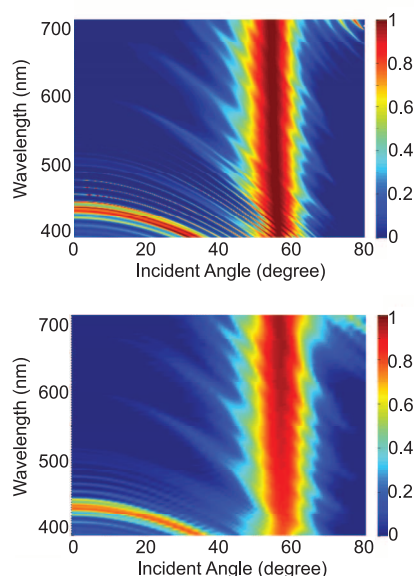


Fig. 4. Simulation and experimental measurement. Comparison between the p-polarized transmission spectrum of the RCWA simulation (27) (top) and the corresponding experimental measurements (bottom). The value of transmission is indicated by the color bars.

was immersed into a colorless liquid with dielectric constant $\epsilon_{\text{liquid}} = \epsilon_1 = 2.18$ (Cargille Labs) (Fig. 3A). The sample could work in the air by adding a coupling prism or by using a porous material for ϵ_1 that has a lower refractive index, such as aerogel (26).

The sample is transparent (up to 98%) to p-polarized incident light at $\theta_B = 55^\circ$ (Fig. 3D); the angular window of transparency is about 8° . It behaves like a mirror at all other incident angles over the entire visible spectrum (Fig. 3, B, C, and E). For s-polarized incident light, the sample behaves like a mirror at all angles (fig. S2) (20). The p-polarization transmittance of the sample in the visible spectrum was measured using an ultraviolet-visible spectrophotometer (Cary 500i); a p-polarizer was used to filter the source beam. The experimentally measured result agrees with the rigorous coupled wave analysis (RCWA) (27) simulation prediction (Fig. 4), which includes the measured dispersion of materials (index variation $< 1.3\%$ for SiO_2 and $< 6.2\%$ for Ta_2O_5 over the wavelength range from 400 to 700 nm). In the experimental measurements, the peak transmittance at θ_B becomes lower at shorter wavelengths (Fig. 4, lower panel) because the wavelength is getting closer to the dimensional tolerance of fabrication. Movie S1 is a video recording of the full process with the sample rotating 90° in this experimental setup.

Our method has a number of attractive features, including simplicity, narrow angle selectivity, scalability beyond optical frequencies, and reproducibility on large scales. Furthermore, this method can be implemented in other wave systems that have Brewster angle analogs, such as acoustic and elastic waves. A natural next step would be to examine materials whose magnetic permeability is similar to their dielectric constant, so as to reach angular selectivity in both polarizations.

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Supplementary Materials

www.sciencemag.org/content/343/6178/1499/suppl/DC1
Figs. S1 to S3
Reference (28)
Movie S1

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Hydrologic Regulation of Chemical Weathering and the Geologic Carbon Cycle

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Earth's temperature is thought to be regulated by a negative feedback between atmospheric CO₂ levels and chemical weathering of silicate rocks that operates over million-year time scales. To explain variations in the strength of the weathering feedback, we present a model for silicate weathering that regulates climatic and tectonic forcing through hydrologic processes and imposes a thermodynamic limit on weathering fluxes, based on the physical and chemical properties of river basins. Climate regulation by silicate weathering is thus strongest when global topography is elevated, similar to the situation today, and lowest when global topography is more subdued, allowing planetary temperatures to vary depending on the global distribution of topography and mountain belts, even in the absence of appreciable changes in CO₂ degassing rates.

Despite substantial changes in solar luminosity, plate tectonics, and atmospheric composition, over billions of years temperatures on Earth have remained favorable for liquid water and, by extension, life (1). A requirement for maintaining such clement conditions is a chemical weathering process that converts atmospheric CO₂ and silicate rocks to alkalinity and divalent cations, which are then buried on the seafloor as carbonate minerals (2–5). Chemical weathering rates cannot be out of balance with the supply of CO₂ from volcanic and metamorphic sources for very long without catastrophic consequences (6). Fortunately, such imbalances have been infrequent. Yet, Earth's climate has varied between warm ice-free conditions and cold extensively glaciated states, suggesting a climate system with variable regulation. The stability of Earth's climate thus requires both a negative feedback between chemical weathering rates and temperature and a mechanism that allows the strength of the feedback, or extent of regulation, to vary (7). The strength of the feedback is dictated by the functional relationship between the weathering rate and climate, and when balanced against CO₂ degassing rates, determines planetary temperatures (4–7). Several processes could allow the strength of the feedback to vary (8–12), suggesting that the mechanisms underlying one of the most profound features in the sculpting of Earth's history remain unresolved.

We developed a mathematical framework that captures how the strength of the weathering feedback changes as a function of the tectonic regime (13). We linked the weathering rate per unit of area of continent to the interaction between runoff and tectonic processes. The two

are linked by the balance between the time that water spends in the weathering zone (fluid travel time), which depends on runoff and flow path length, and the kinetics of mineral weathering, which are a function of composition, temperature, and erosion rate. The weathering flux to the oceans should be maximized when thermodynamic equilibrium between the dissolving and precipitating minerals is approached, resulting in a “thermodynamic limit” not included in previous models. Conversely, weathering fluxes are at a minimum when fluid travel times are short relative to the time required for weathering reactions to reach equilibrium (equilibrium time). In this formulation, weathering

rates can increase more substantially in tectonically active than in inactive areas in response to changes in climate, as suggested by numerical models (7, 14).

The proposed model combines two equations: (1) a solute transport equation that quantifies weathering-derived solute as a function of the mean fluid travel time in a catchment and (2) an equation that relates the supply of fresh rock from erosion to the downward propagation of a weathering front. We calculate the solute concentration (C) as a function of the Damköhler number (Da) (15). The Damköhler number is a dimensionless number that compares the mean fluid travel time [$T_f \approx L\phi/q(\text{year})$] to the time required to reach equilibrium [$T_{eq} \approx C_{eq}/R_n(\text{year})$], where $q[\text{m/year}]$ is runoff, the reactive flowpath length ($L\phi$) is flowpath length [$L(\text{m})$] times effective porosity (ϕ), $C_{eq}(\mu\text{mol/liter})$ is the “thermodynamic limit” (i.e., the maximum concentration), and $R_n(\mu\text{mol/liter/year})$ is the reaction rate (n , a specific mineral composition) (16). Because runoff is a key variable, we factor out runoff and introduce the Damköhler coefficient (Dw), which is modified to account for the supply of fresh minerals through erosion.

$$Dw(\text{m/year}) = \frac{L\phi}{T_{eq}} = \frac{L\phi R_{n,\text{max}} f_w}{C_{eq}} \quad (1)$$

where the fraction of fresh minerals, $f_w (= X_s/X_r)$, decreases with increasing soil age (T_s) or decreasing mineral supply rate (17–20) to moderate the maximum reaction rate ($R_{n,\text{max}}$). The minimum T_{eq} occurs when the concentration of soil minerals (X_s) is equal to that of the original rock (X_r), or when T_s is very small, according to the

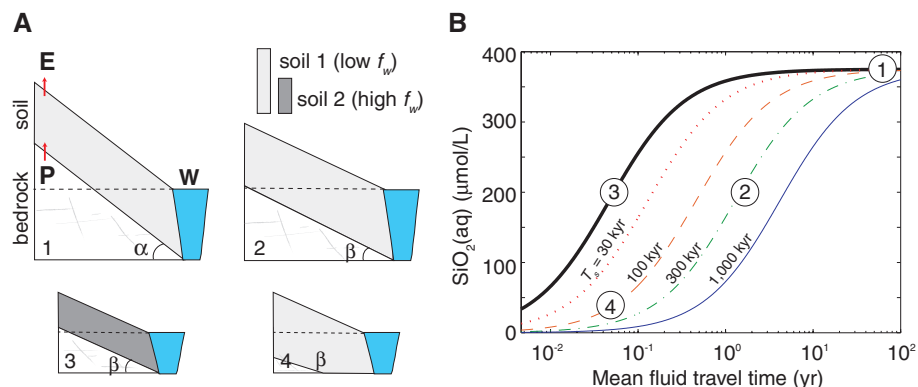


Fig. 1. Conceptual model for solute production in catchments. (A) Hillslope cross-sections with variable slope, soil thickness, mineral abundance, and mean fluid travel time. Red arrows show the production of soil from bedrock (P) and soil removal by erosion (E) and chemical denudation (W). Dashed lines indicate the water table, whereas slope is distinguished by angles α and β , with $\alpha > \beta$. Soil thickness and f_w are equivalent for hillslopes 1, 2, and 4, whereas soil thickness is lower and f_w higher for hillslope 3. Fresh bedrock above the water table decreases the T_{eq} , so that $T_f > T_{eq}$. (B) Evolution of $\text{SiO}_2(\text{aq})$ as a function of T_f and T_s . The solid black line corresponds to weathering of fresh mineral, whereas light and stippled lines correspond to solute evolution for different T_s values. kyr, thousand years. Hillslope 1 (point 1) indicates a system at the thermodynamic limit where $T_f > T_{eq}$, as compared to hillslopes 2 to 4, where $T_f < T_{eq}$. Hillslopes 3 and 4 show how mineral supply (or f_w) affects solute production, hillslopes 2 and 4 show how T_f affects solute production, and the greater relief of hillslope 1 as compared to that of 2 results in larger $L\phi$.

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following expression that is applicable to both steady-state (i.e., a balance between denudation and material supply) and transient landscapes

$$f_w(g/g) = \frac{X_s}{X_r} = \frac{1}{1 + mk_{\text{eff}}AT_s} \quad (2)$$

where k_{eff} is the weathering rate constant, A is the specific surface area of the minerals, and m is the molar mass of the weathering minerals [(13), table S1]. At a given runoff, larger Dw values reflect more-efficient solute generation and systems that are closer to the thermodynamic limit. The final equation for solute production is

$$C(\mu\text{mol/L}) = C_{\text{eq}} \frac{\tau Dw/q}{1 + \tau Dw/q} \quad (3)$$

where τ is a constant ($= e^2$) [(13), figs. S1 to S3]. The weathering flux (W) is computed from Eq. (3) as $C \times q$. Although we use $\text{SiO}_2(\text{aq})$ as a proxy for weathering, Ca and Mg derived from silicate weathering would show the same behavior (13, 16). The solute production model does not capture all possible biogeochemical and climatic feedbacks that might operate on q , C_{eq} , k_{eff} , and T_s (3–6, 11). Some key parameters are also not well constrained by observations, and hence our assumptions require further evaluation as suitable data become available (21).

The maximum solute concentration occurs when the mean fluid travel time (T_f) exceeds the equilibrium time (T_{eq}) at a given runoff. For example, consider a region on Earth at point 4 on Fig. 1, with short T_f and long T_{eq} (22). If fresh minerals are supplied to the weathering zone, T_{eq} decreases and solute concentrations increase,

even with no change in T_f (point 3). As erosion increases and T_{eq} becomes less than T_f , the thermodynamic limit is approached (point 1) and solute concentration remains fixed. At the thermodynamic limit, concentration does not vary with runoff, as observed for some rivers (23, 24). As declining surface uplift or erosion decreases the supply of fresh minerals from soil production, T_{eq} will increase, resulting in lower solute concentration, even if T_f remains long (point 2). Hence, climate (through runoff) is mechanistically coupled to erosion through the operative physical length scales and thermodynamic limits.

This formulation requires that as T_f becomes shorter than T_{eq} , solute concentrations decrease and solute fluxes plateau (Fig. 2). The Dw determines the runoff at which this plateau is reached. This relationship is observed in modern rivers draining active mountain ranges where high erosion and a rapid supply of fresh minerals result in short T_{eq} , so that solute concentrations and fluxes are high (Fig. 2). These systems have Dw values greater than 0.03 m/year. In contrast, in tectonically inactive areas, such as large cratons, the solute concentrations and fluxes are smaller and Dw values lower, because the reduced supply of fresh minerals to the weathering zone increases T_{eq} (25). The competition between T_f and T_{eq} in modern river basins is evident by the large range of Dw values—about a factor of 100. Although we use $\text{SiO}_2(\text{aq})$ as a proxy for weathering, Ca and Mg derived from silicate weathering would show the same behavior (16).

To change solute fluxes in rivers requires altering $L\phi$, T_{eq} , and/or runoff via tectonic and/or climatic events. For example, the construction of a mountain belt will alter all three of these pa-

rameters through the development of relief, an increased supply of fresh minerals (26, 27), and increased runoff due to orographically induced precipitation (28). During initial mountain building, fresh rock exposed to the weathering zone shortens T_{eq} and increases solute concentrations (Fig. 3A). When mountains reach sufficient height to create orographic rainfall, solute fluxes will increase further with additional runoff. Even if thin soils result in short T_f in upland areas, the creation of upland topography may result in the deposition of reactive floodplains (29, 30), a decrease in T_{eq} , and possibly an increase in T_f . Once the mountain belt reaches a physical steady state, no additional increase in chemical weathering is possible without a change in runoff. Finally, as tectonism wanes, reducing mineral supply to the weathering zone, solute fluxes decrease and complete the cycle.

The atmospheric heat budget of Earth is driven by the fluxes of atmospheric greenhouse gases, primarily CO_2 . Silicate weathering is the dominant mechanism that sequesters CO_2 over geologic time scales (2). We propose that the removal of CO_2 is regulated by the intensity of the hydrologic cycle, rather than directly by temperature. Increases in global mean temperatures (GMTs) are associated with more precipitation and attendant changes in runoff (31), although climate model predictions vary widely. A climate model study of the response of runoff to increased GMT found an increase in runoff of 6.8 to 2.3%/°C between high- and low-latitude rivers, respectively (31). Because the effects of global warming on runoff will not be distributed evenly across latitudes, we use the maximum runoff sensitivity of 6.8%/°C GMT, so that a doubling of

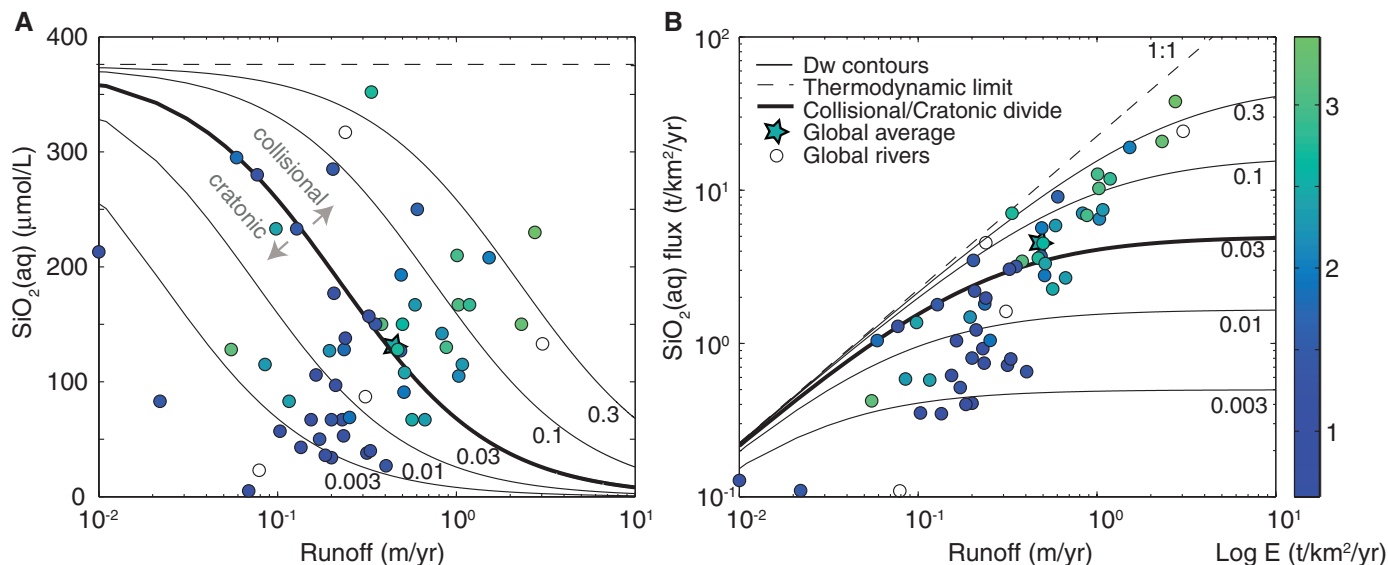
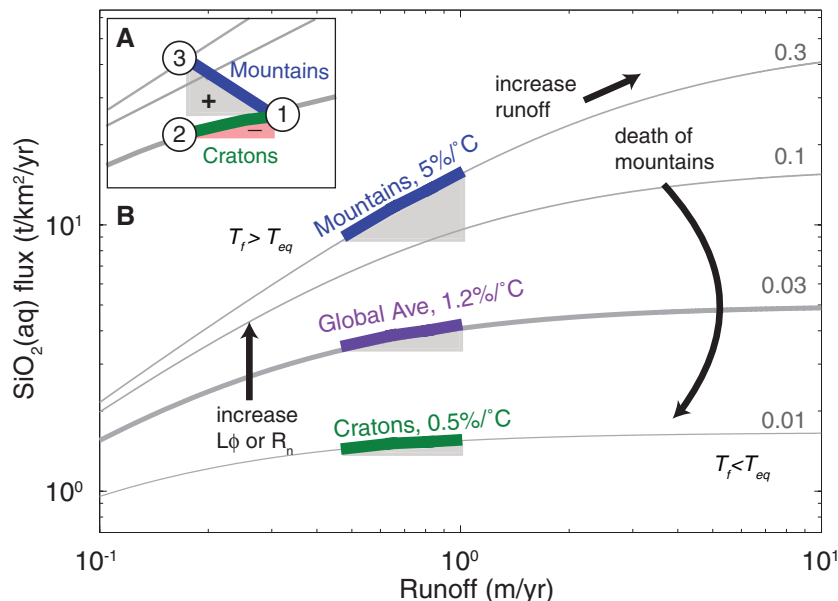


Fig. 2. Contours of global Damköhler coefficients applied to large rivers. (A) $\text{SiO}_2(\text{aq})$ for global rivers (δ) as a function of runoff, compared to contours of Dw coefficients from 0.3 and 0.001 m/year, where 0.3 m/year corresponds to the global maximum Dw and C_{eq} . **(B)** $\text{SiO}_2(\text{aq})$ fluxes. The range of Dw contours corresponds to either changes in T_s from 0 to 1 million

years, $L\phi$ of 0.10 to 0.001 m, or variations in both. A Dw of 0.03 m/year (collisional/cratonic divide) corresponds to $L\phi$ of 0.1 m and T_s of 100,000 years. The 1:1 line for flux:discharge is the thermodynamic limit. Each river is shaded according to the erosional flux (assumed to reflect mineral supply to the weathering zone), using the total suspended sediment flux.

Fig. 3. Sensitivity of weathering fluxes to changes in GMT. (A) The inset depicts how an enhanced supply of fresh mineral from increasing erosion, combined with global cooling and decreased runoff, results in a decrease in weathering fluxes from the cratons (points 1 to 2, minus sign, pink wedge), whereas solute fluxes increase in mountains (points 1 to 3, plus sign, gray wedge), so that weathering fluxes (and global CO_2 levels) could remain at near-modern levels, depending on the area occupied by different weathering regimes. (B) Colored bars and gray wedges show the change in solute flux for a doubling of runoff for mountainous regions (blue), the global average (purple), and cratons (green), with the corresponding percent change in solute flux per $^\circ\text{C}$ of increase. Gray lines are contours of Dw as in Fig. 2.



runoff occurs with a temperature increase of 15°C . Assuming that temperature affects only runoff (supplementary text and figs. S7 and S8), changes in solute fluxes for a doubling of runoff across low-relief regions, active tectonic regions, and the global average indicate the differential climate sensitivity (Fig. 3B). Weathering fluxes in basins with moderate erosion rates, which represent a large portion of Earth's surface today (32), show minimal change. In contrast, active high-relief regions near the thermodynamic limit are the most sensitive to climatic changes (27), because higher Dw values afford a large change in runoff before maximum solute flux is achieved.

The order-of-magnitude difference in climate sensitivity between inactive and active tectonic regions may dramatically alter the global weathering feedback. The climate-weathering feedback is strongest in rapidly eroding regions near the thermodynamic limit, as shown by the wedges in Fig. 3B. In low-relief areas, the climate feedback is weaker, because solute fluxes plateau when T_{eq} exceeds T_f . During times of global warming and amplification of the hydrologic cycle, weathering fluxes will increase disproportionately between tectonically active and inactive areas, with high-relief tectonic areas providing proportionally more alkalinity to the oceans. At a given CO_2 degassing rate, global temperature is thus determined by the differential sensitivity of mountainous and low-relief areas to runoff, and will vary depending on the distribution of erosional regimes and mountain belts (Fig. 3A). This feature of our model allows weathering rates and atmospheric CO_2 to stabilize at different levels but prevents runaway CO_2 consumption during active tectonic periods, due to the enhanced climate sensitivity. Similarly, periods of warming and the attendant increases in runoff will drive weathering rates upward, particularly in moun-

tainous regions. The solute production model explains the observed correlation between erosion and chemical weathering fluxes (8, 26), while preserving the negative feedback between silicate weathering rates and global temperature.

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- Independent calculation of Dw coefficients via Eq. 1 would be one approach. At present, sufficient data for large rivers are not available.
- To compute the curves in Figs. 1 to 3, we use the concentration and discharge data of (8), $R_{\text{p,max}}$ of $1085 \mu\text{mol/liter/year}$, and a maximum C_{eq} of $375 \mu\text{mol/liter}$ (table S1) to determine the fluxes and Dw contours associated with the global rivers, mostly draining granitic lithologies. Basaltic lithologies are expected to follow the same general behavior (fig. S8). In Fig. 1, we show contours of T_s corresponding to Dw contours in Figs. 2 and 3. The contours of Dw , which do not depend on the absolute values of R_n , f_w , or T_s , illustrate how rivers respond in a relative sense to climatic and tectonic variations.
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Contribution of NAC Transcription Factors to Plant Adaptation to Land

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The development of cells specialized for water conduction or support is a striking innovation of plants that has enabled them to colonize land. The NAC transcription factors regulate the differentiation of these cells in vascular plants. However, the path by which plants with these cells have evolved from their nonvascular ancestors is unclear. We investigated genes of the moss *Physcomitrella patens* that encode NAC proteins. Loss-of-function mutants formed abnormal water-conducting and supporting cells, as well as malformed sporophyte cells, and overexpression induced ectopic differentiation of water-conducting-like cells. Our results show conservation of transcriptional regulation and cellular function between moss and *Arabidopsis thaliana* water-conducting cells. The conserved genetic basis suggests roles for NAC proteins in the adaptation of plants to land.

The acquisition of water-conducting tissue enabled the transition of plants from an aqueous environment to land. To achieve this, vascular plants developed xylem vessel elements, cells that have a characteristic thickened secondary cell wall and undergo programmed cell death at maturity. These cells provide mechanical strength to the stem, while allowing efficient water conduction. Recent work revealed that a group of NAC [no apical meristem (NAM), *Arabidopsis* transcription activation factor (ATAF1/2), and cup-shaped cotyledon (CUC)] transcription factors, including VASCULAR-RELATED NAC-DOMAIN6 (VND6) and VND7 of *Arabidopsis thaliana*, regulate xylem vessel differentiation by inducing genes for secondary cell wall biosynthesis and xylem vessel-specific programmed cell death (1–6). NAC proteins also regulate the development of fiber cells: sclerenchyma cells found in vascular plants, which are also characterized by a thickened secondary wall (7–10). In *A. thaliana*, NAC SECONDARY WALL THICKENING PROMOTING FACTOR (NST)/SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN (SND) proteins, constituting a sister group to VND, regulate fiber cell differentiation, suggesting an evolutionary relationship between vessels and fibers (7, 8). Thus, conservation of a VND/NST/SND-based

transcriptional regulatory system for secondary wall biosynthesis has been proposed (11).

In mosses, specialized cell types have been characterized. For example, hydroid cells conduct water internally (12, 13), and mature hydroids

share several characteristics with xylem vessels from vascular plants, such as an elongated shape and an absence of cellular contents (13). However, unlike xylem vessels, hydroids lack distinct pits on their lateral walls, and there is an absence of lignified secondary wall (14–17). Stereoid cells have thickened cell walls and are hypothesized to function as supporting elements (18). Both hydroids and stereoids are formed mainly in the gametophytic generation, unlike xylem vessels and fiber cells, which are formed only in the sporophytic generation of vascular plants. Therefore, it remains unclear whether hydroids and stereoids share an evolutionary lineage with xylem vessels and fiber cells, respectively.

The moss *P. patens* genome has eight loci that share similarity with VND/NST/SND (19–21). We sequenced cDNA from these genes and named the gene family *PpVNS* [*VND*-, *NST*/*SND*-, *SMB* (*SOMBRERO*)-related protein], with genes 1 through 8 (22) (fig. S1 and table S1). For phylogenetic analysis, publicly available genomic and transcriptomic sequence data were searched. Although we identified a single gene of *Marchantia polymorpha*,

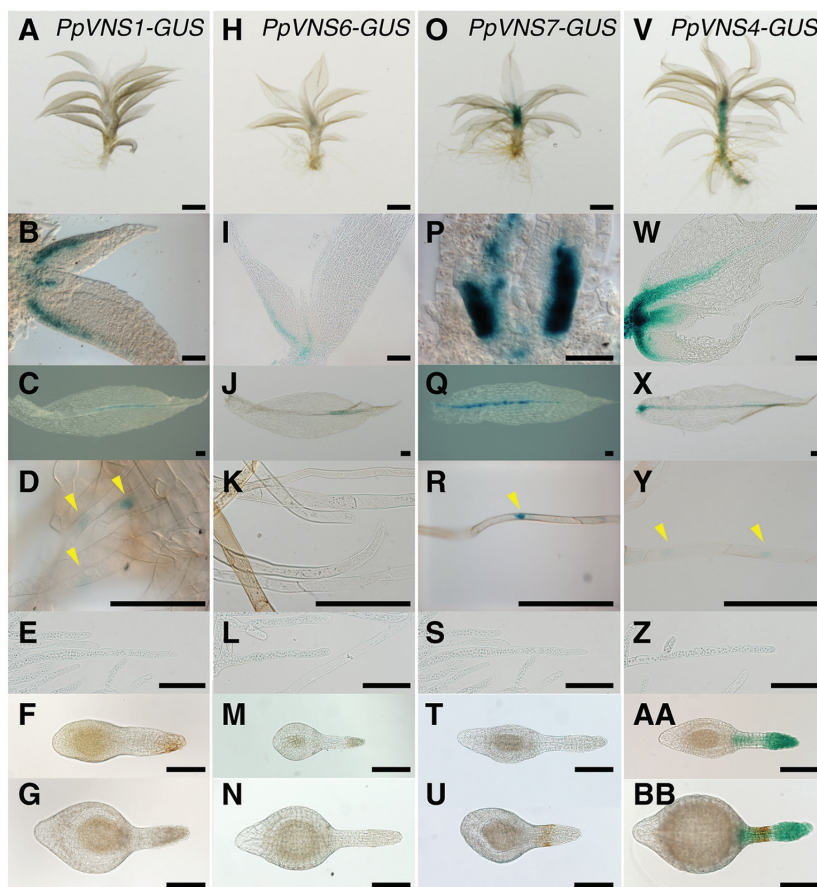


Fig. 1. Expression of *PpVNS* genes in *P. patens* tissues. GUS expression pattern of *PpVNS1-GUS* (A to G), *PpVNS6-GUS* (H to N), *PpVNS7-GUS* (O to U), and *PpVNS4-GUS* (V to BB) reporter lines. The central region of young leaves [(B), (I), (P), and (W)], midribs [(C), (J), (Q), and (X)], rhizoids [(D), (K), (R), and (Y), yellow arrowheads], protonemata [(E), (G), (L), (N), (S), (U), (Z), and (BB)], and sporophytes [(F), (M), (T), and (AA)], early stages; (G), (N), (U), and (BB), late stages] are shown. Scale bars, 5 mm in (A), (H), (O), and (V); 100 μ m in (B) to (E), (I) to (L), (P) to (S), and (W) to (Z); and 200 μ m in (F), (G), (M), (N), (T), (U), (AA), and (BB).

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P. patens, *Selaginella moellendorffii*, *A. thaliana*, poplar, and rice all had many (fig. S2), suggesting an early expansion of *VNS* genes in prevascular

plants (20, 21). We examined the tissue-specific expression patterns of *PpVNS* by quantitative reverse transcription polymerase chain reaction (qRT-PCR)

in tissues from protonemata and gametophytes. In the gametophytic generation, after haploid spore germination, *P. patens* forms filamentous cells called

Fig. 2. Defects in midrib development in the *ppvns1 ppvns6 ppvns7* mutant. (A to C) Transport of Evans Blue dye in the leaves of wild-type (WT) plants (A) and in *ppvns1 ppvns6 ppvns7* line 8 (B) and line 35 (C) after 30 min of incubation. Arrowheads indicate positions where Evans Blue dye was transported from the base. (D to I) Typical images of transmission electron microscopy transverse sections of the tip [(D) and (G)], middle [(E) and (H)], and basal [(F) and (I)] regions of leaves of wild-type plants [(D) to (F)] and *ppvns1 ppvns6 ppvns7* [(G) to (I)]. h, e, and s indicate content-free hydroid cells with thin cell walls, epidermal cells with developed chloroplasts, and stereid cells with thicker cell walls, respectively. (J to L) Images of wild-type plants (J) and mutants [(K) and (L)] incubated for 8 hours under the low-humidity condition. Wilted leaves were observed in the mutant. Scale bars, 200 μ m in (A) to (C); 5 μ m in (D) to (I); and 500 μ m in (J) to (L).

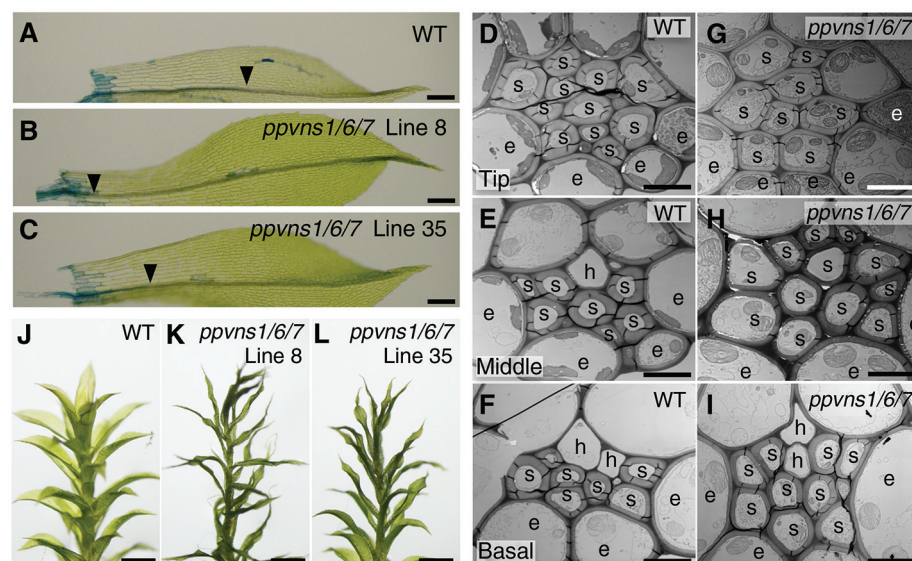
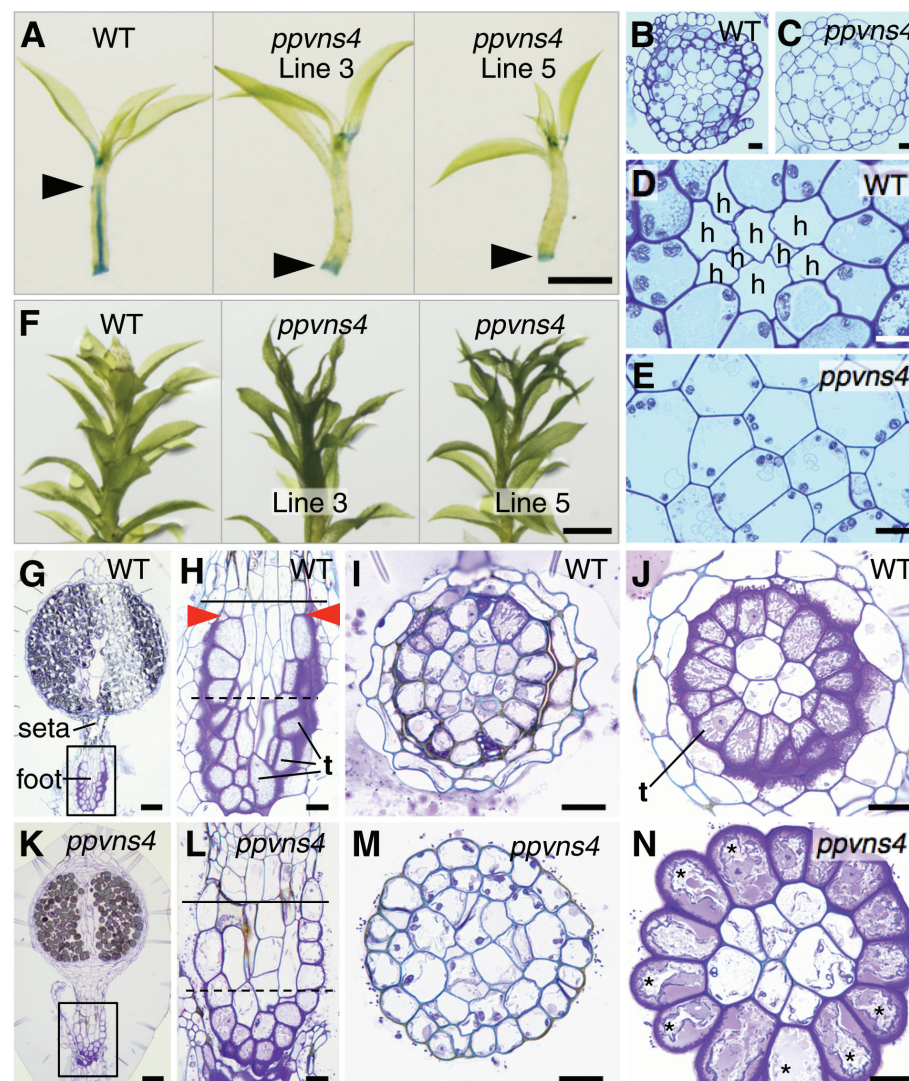


Fig. 3. Defects in stem and sporophyte development in the *ppvns4* mutant. (A) Transport of Evans Blue dye through stems of wild-type (WT) plants and *ppvns4* deletion mutants after 30 min of incubation. Black arrowheads indicate positions where Evans Blue dye was transported from the base. (B to E) Microscopic images of stem sections of wild-type plants [(B) and (D)] and *ppvns4* mutants [(C) and (E)]. (D) and (E) Magnified views are shown in (B) and (C), respectively. The h's in (D) indicate cytoplasmic content-free hydroid cells with a thin cell wall in the central region of wild-type plants. (F) Typical images of wild-type plants and *ppvns4* mutants incubated for 8 hours under the low-humidity condition. Wilted leaves were observed in *ppvns4*. (G to N) Matured sporophytes of wild-type plants [(G) to (J)] and *ppvns4* mutants [(K) to (N)]. (H) and (L) Magnified views of the regions enclosed in black squares in (G) and (K), respectively. Red arrowheads indicate the boundary between the seta and the foot. Thin solid lines and dotted lines in (H) and (L) indicate the position of transverse section shown in (I) and (M), and (J) and (N), respectively. t indicates transfer cells. Asterisks indicate malformed vacuoles (N). Archegonium cells surrounding a sporophyte remain in (G) to (J). Scale bars, 1 mm in (A); 20 μ m in (B) to (E), (H) to (J), and (L) to (N); 500 μ m in (F); and 80 μ m in (G) and (K).



protonemata, from which leafy gametophores develop. Although the expression of *PpVNS3* was barely detected in the tested tissues, the other seven *PpVNS* genes were all expressed, and they were more abundant in gametophores than in protonemata (fig. S3, A and B). The *PpVNS-GUS* (β -glucuronidase) reporter lines (22) (fig. S4), in agreement with qRT-PCR results, showed

that all *PpVNS* genes were preferentially expressed in gametophores, except for *PpVNS3-GUS*, whose signal was too low to be detected in any tissue (Fig. 1, A to BB, and fig. S3). *PpVNS-GUS* activity was prominent in the newly emerged leaf central region (which is thought to subsequently develop into the midrib) and in the developing leaf midrib, which is composed of several types

of differentiated cells, including hydroids and stereids (Fig. 1 and figs. S3 and S5 to S7), suggesting that *PpVNS* genes function in midrib development. *PpVNS1*, *PpVNS6*, and *PpVNS7* were preferentially expressed in these regions (table S2), whereas the other *PpVNS* genes were also expressed in other regions of the leaf (fig. S5 and table S2). GUS signals were also detected in stems

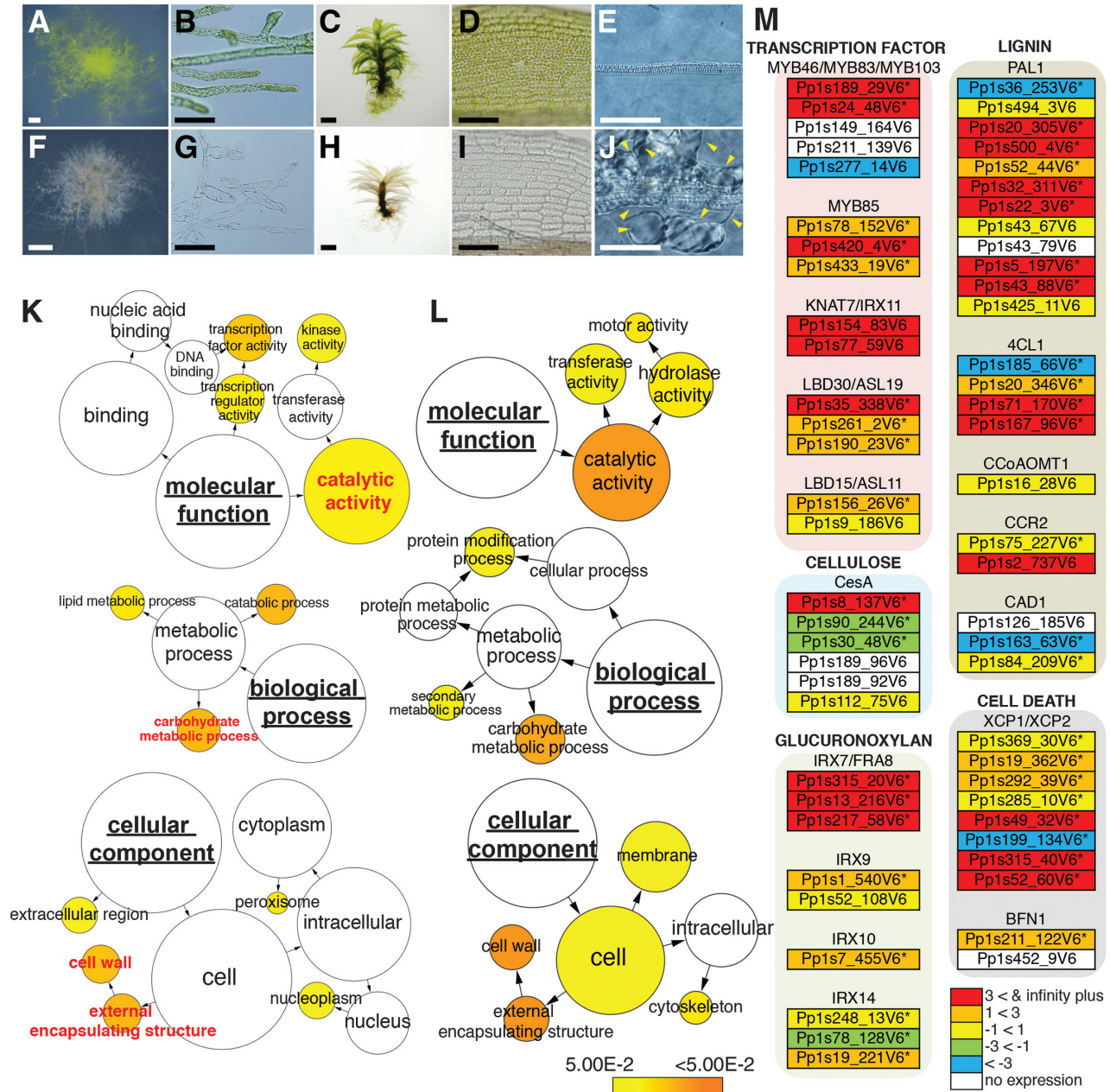


Fig. 4. Effects of estrogen-induced overexpression of *PpVNS7* genes in *P. patens* and *A. thaliana*. Phenotypes shown in protonemata and gametophores of *P. patens* (A to D, F to I) and *A. thaliana* leaves (E and J) treated with 1 μ M 17- β -estradiol (ER), wild-type ER-WT [(A) to (E)], and the *PpVNS7* overexpressor ER-*PpVNS7* [(F) to (J)]. (B) and (G) Magnified views shown in (A) and (F), respectively. (D) and (I) Leaves from gametophores shown in (C) and (H), respectively. Yellow arrowheads indicate ectopic secondary cell wall deposition. (K and L) Enriched gene ontology (GO) terms for the up-regulated 2910 *P. patens* genes in the *PpVNS7* overexpressor (K) and the reported 448 VND/NST/SND direct target genes in *A. thaliana* (L). Node size reflects the

number of genes belonging to the category. Colored nodes represent GO terms that are significantly overrepresented (corrected P value < 0.05), and the color scale indicates increasingly higher statistical significance. Overrepresented GO terms detected commonly between *P. patens* and *A. thaliana* are indicated in red characters (left). (M) Changes in the expression level of *P. patens* genes that are putatively orthologous to well-characterized *A. thaliana* genes involved in cell wall biosynthesis and cell death. The color scale indicates fold changes of gene expression (on a log 2 scale). Asterisks indicate statistically significant changes. Scale bars, 500 μ m in (A) and (F); 100 μ m in (B), (D), (G), and (I); 1 mm in (C) and (H); and 50 μ m in (E) and (J).

(only *PpVNS4*), rhizoids (except for *PpVNS3* and *PpVNS6*), and protonemata (*PpVNS2*, *PpVNS5*, and *PpVNS8*) (fig. S3 and table S2). Thus, the *PpVNS* genes function in the development of other tissues in addition to that of the midrib.

Because *PpVNS1*, *PpVNS6*, and *PpVNS7* are prominently expressed in the midrib (Fig. 1 and table S2), we investigated their function in midrib development. To examine water transport in the triple deletion mutant (*ppvns1 ppvns6 ppvns7*) lines (22) (fig. S8), excised leaves were soaked in Evans Blue dye, which is absorbed and transported from the cut edges of the leaf base through the midrib (Fig. 2, A to C). The distance of dye penetration was reduced in the mutants as compared with the wild type (fig. S9, A and B), suggesting that water transport is deficient in the mutants. Although leaves of mutants had hydroid cells at the leaf base (Fig. 2I), there were no typical hydroid cells in the middle parts of leaves; such cells as were there had abnormally thickened walls and occasionally retained some cellular contents (Fig. 2H). Therefore, the decreased water transport activity observed in the triple mutants could be attributed to the malformation of hydroid cells. Reflecting this deficient water transport activity, under low-humidity conditions, *ppvns1 ppvns6 ppvns7* leaves were more prone to wilting (22) (Fig. 2, J to L, and table S3). *ppvns1 ppvns6 ppvns7* showed abnormalities not only in hydroids but in sterioids (Fig. 2, G to I). Stereoid cell wall thickness was reduced in the triple mutants to about less than half of that in wild type (22) (table S4 and fig. S10, A and B). Sterioids of the triple mutants retained cellular contents (Fig. 2, G to I), unlike wild-type sterioids (Fig. 2, D to F), and their sizes were up to approximately 1.2 times as large as the wild type (fig. S10D). Closely related hydroid phenotypes were observed in the stems of single deletion mutant lines for *PpVNS4*, which is the only gene with the detectable GUS signals in the stems of gametophores (Fig. 1V). *ppvns4* stems did not transport the dye (Fig. 3A and fig. S9C) and had immature hydroid cells; cells located at the positions of hydroids had cellular contents including plastids (Fig. 3, B to E), with the leaf wilting phenotype (Fig. 3F and table S3). In addition, *ppvns4* showed abnormality in sporophyte development. Although typical hydroid cells were not observed even in wild-type sporophytes, both the central and transfer cells of the foot, where *PpVNS4* is preferentially expressed (Fig. 1, AA and BB), were enlarged in *ppvns4*, and the organization of vacuoles and plastids of these cells was abnormal (22) (see supplementary note 1 for the details; Fig. 3, G to N, and fig. S11). Thus, our data suggested critical roles for PpVNS in the development of specialized types of cell in *P. patens*: *PpVNS1*, *PpVNS6*, and *PpVNS7* control the development of both hydroids and sterioids in the gametophyte leaf, and *PpVNS4* controls the development of hydroids in the gametophyte stem and of central and transfer cells in the sporophyte foot.

Next, we overexpressed the *PpVNS* genes in *P. patens* and *A. thaliana* by using the estrogen-

inducible overexpression system (22) (fig. S12). In *P. patens*, overexpression of the *PpVNS* genes, as well as of the *A. thaliana* *VND7*, induced cell death in both protonemata and gametophores (Fig. 4 and figs. S13 and S14). The cell death response to *PpVNS* overexpression included degradation of chloroplasts, shrinkage of protoplasm away from the cell wall, and loss of plasma membrane integrity (as revealed by Evans Blue staining) (fig. S14), and its progress was faster than the cell death induced by stresses such as high temperature and ultraviolet irradiation (fig. S15). These suggest that the PpVNS family plays a role in programmed cell death in *P. patens*. RNA-seq analysis showed that overexpression of *PpVNS7* changed expression profiles in *P. patens*, with a selective up-regulation of transcription factors and cell wall biosynthesis genes (see supplementary note 2 for the details; Fig. 4K, tables S5 to S8, and figs. S16 to S18). Detailed analysis revealed that *PpVNS7* can regulate many putative orthologous genes to the VND/NST/SND direct targets, such as *MYB46/83/103*; transcriptional activators for secondary wall formation (5, 23); *CesA*, a cellulose synthase subunit gene (24); *IRX7/FRA8*, a glucuronoxylan biosynthesis gene (25); *4CL*, a lignin biosynthesis gene (26); and *XCP*, a papain-like cysteine peptidase gene (3) (Fig. 4, L and M, and tables S9 and S10), suggesting an evolutionary conservation of VNS downstream genes. In *A. thaliana*, *PpVNS* overexpression led to ectopic secondary wall deposition (Fig. 4, E and J, and fig. S19), similar to that observed when *A. thaliana* or poplar *VNS* genes were overexpressed in *A. thaliana* (2, 6–10). qRT-PCR analysis revealed that *PpVNS* overexpression up-regulated *MYB46* in addition to secondary wall-specific *CesA* genes (24) and *XCP1* (3) in *A. thaliana* (fig. S20). Thus, the overexpression of *PpVNS* genes promotes the expression of genes that support water-conducting cell formation, and those developmental pathways lead to ectopic cell wall thickening and programmed cell death in both *P. patens* and *A. thaliana*.

Our results demonstrate that PpVNS proteins regulate hydroid cell differentiation by inducing their death. PpVNS proteins also regulate stereoid cell differentiation by promoting cell wall thickening and the clearance of cellular contents. PpVNS regulates the same gene families in both mosses and vascular plants, and thus a genetic basis for the formation of both water-conducting and supporting tissue is conserved among them. Mosses develop hydroids and sterioids in the gametophytic generation; vascular plants establish the vascular system in the sporophytic generation. Together, it seems that the VNS-based gene regulatory networks in the gametophyte and sporophyte generations are homologous and are elaborated in the sporophyte generation during vascular plant evolution.

Water conductivity through dead cells is much higher than that through living parenchyma cells (27). Thus, the evolution of the cell death program may have facilitated water conduction. Likewise, supporting elements are required to produce large, complex plant bodies. The NAC transcription fac-

tors may have contributed to the evolution of both water-conducting and supporting cells during the adaptation of plants to land.

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Supplementary Materials

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A Bacterial Tyrosine Phosphatase Inhibits Plant Pattern Recognition Receptor Activation

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Innate immunity relies on the perception of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) located on the host cell's surface. Many plant PRRs are kinases. Here, we report that the *Arabidopsis* receptor kinase EF-TU RECEPTOR (EFR), which perceives the elf18 peptide derived from bacterial elongation factor Tu, is activated upon ligand binding by phosphorylation on its tyrosine residues. Phosphorylation of a single tyrosine residue, Y836, is required for activation of EFR and downstream immunity to the phytopathogenic bacterium *Pseudomonas syringae*. A tyrosine phosphatase, HopAO1, secreted by *P. syringae*, reduces EFR phosphorylation and prevents subsequent immune responses. Thus, host and pathogen compete to take control of PRR tyrosine phosphorylation used to initiate antibacterial immunity.

Many plant pattern recognition receptors (PRRs) are receptor kinases, such as the *Arabidopsis* FLS2 and EFR, which recognize the bacterial pathogen-associated molecular patterns (PAMPs) flagellin (or flg22) and elongation factor Tu (EF-Tu) (or elf18), respectively (1). Both FLS2 and EFR belong to the non-RD group of kinases (2) and are important for antibacterial immunity (1). Ligand binding to FLS2 or EFR induces

their association with the receptor kinase BAK1, and reciprocal phosphorylation ensues, which initiates immune signaling (3). The exact phosphorylation events occurring within these complexes and their biological roles are, however, still unknown. Plant receptor kinases have features of Ser-Thr kinases (4). Although tyrosine (Tyr) phosphorylation of receptor kinases is widely studied in mammals (5), little is known about its role in plant signaling.

Recent studies revealed the involvement of Tyr phosphorylation in RD kinases signaling such as for the receptor kinases BRI1 and BAK1 during growth and for the cytoplasmic kinase BIK1 in immunity (6–9). However, nothing is known about the importance of Tyr phosphorylation in non-RD kinases, despite its being the major kinase subclass involved in immune signaling across kingdoms (2).

To test the relevance of Tyr phosphorylation for plant innate immunity, we pretreated *Arabidopsis*

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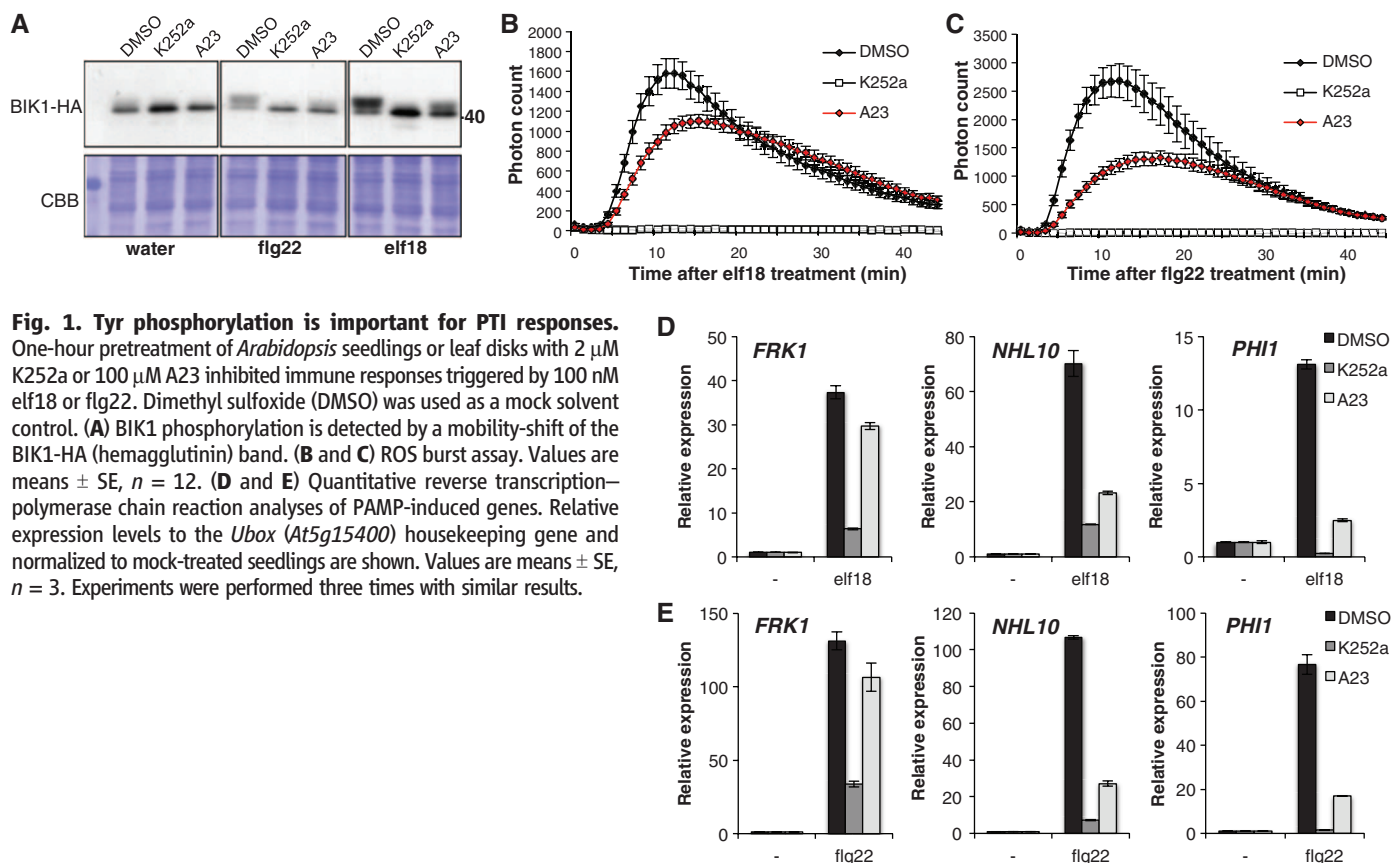
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seedlings with the Tyr kinase inhibitor tyrphostin A23 (A23) and assayed typical rapid immune responses. A23 reduced BIK1 phosphorylation and the burst of reactive oxygen species (ROS) normally observed upon PAMP perception (1, 10, 11) (Fig. 1, A to C). Similarly, A23 reduced the flg22-

and elf18-triggered induction of immune-related genes, such as *FRK1*, *NHL10*, and *PHI1* (Fig. 1, D and E). Other Tyr kinase inhibitors, such as tyrphostin A25 and genistein, also reduced elf18-triggered ROS burst (fig. S1). Pretreatment with the general kinase inhibitor K252a suppressed these

responses (Fig. 1, A to E). Together, these results suggest that Tyr phosphorylation regulates immune signaling induced by PAMPs.

Next, we tested whether Tyr phosphorylation occurs at the level of non-RD kinase PRRs. We focused on EFR, which is a stronger kinase than

Fig. 2. EFR is phosphorylated on Tyr residues. (A) Recombinant MBP (maltose-binding protein)-EFR phosphorylates on Tyr residues in vitro. EFR*, kinase-dead version (D849N). CD, cytoplasmic domain. (B) Tyr phosphorylation on EFR-green fluorescent protein (GFP) immunoprecipitated from *N. benthamiana* after treatment with water (–) or 100 nM elf18 (+) for 10 min. (C) Tyr phosphorylation on EFR-GFP immunoprecipitated from *Arabidopsis* seedlings after elicitation with 100 nM elf18. Immunoblots were analyzed with antibodies against phosphorylated tyrosine (anti-pTyr) or anti-GFP. Full anti-pTyr blot is shown in fig. S3. (D) Treatment with kinase inhibitors abolishes EFR phosphorylation. *Arabidopsis* seedlings were pretreated for 1 hour with 2 μ M K252a or 100 μ M A23 before treatment with water (–) or 100 nM elf18 (+) for 10 min. DMSO was used as a mock solvent control. Immunoprecipitated proteins were incubated with [32 P]-ATP (adenosine 5'-triphosphate). CBB, Coomassie brilliant blue. In vitro phosphorylation was revealed by autoradiography. Experiments were performed three times with similar results.

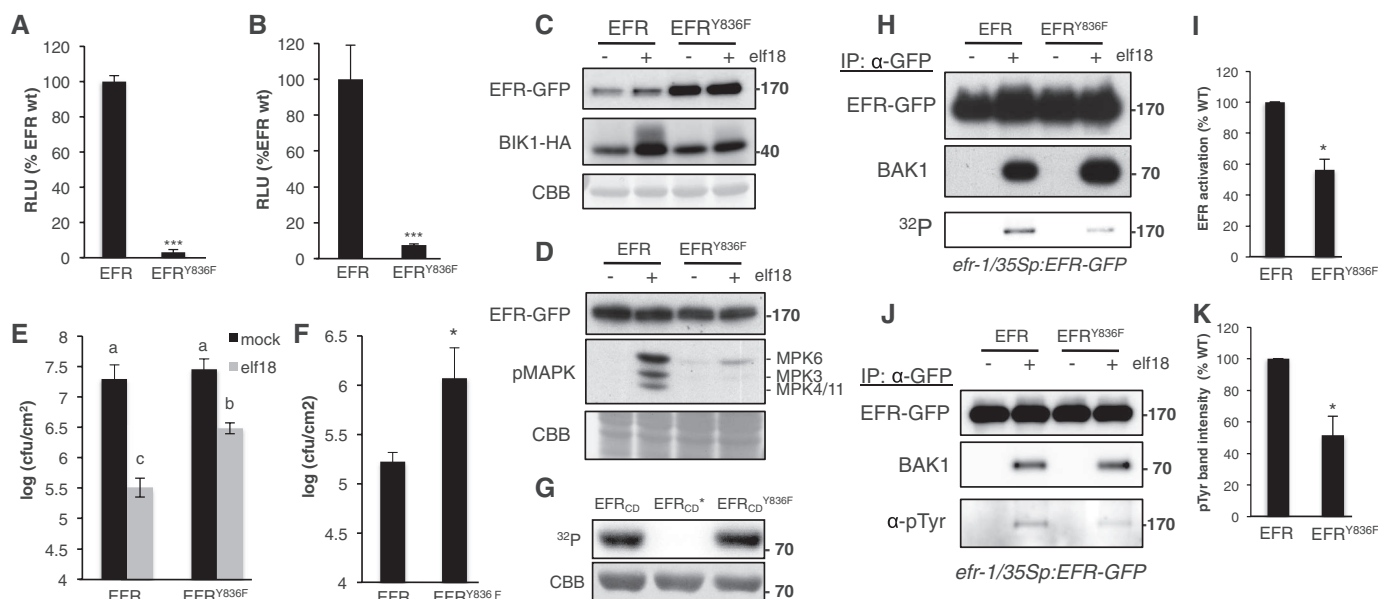
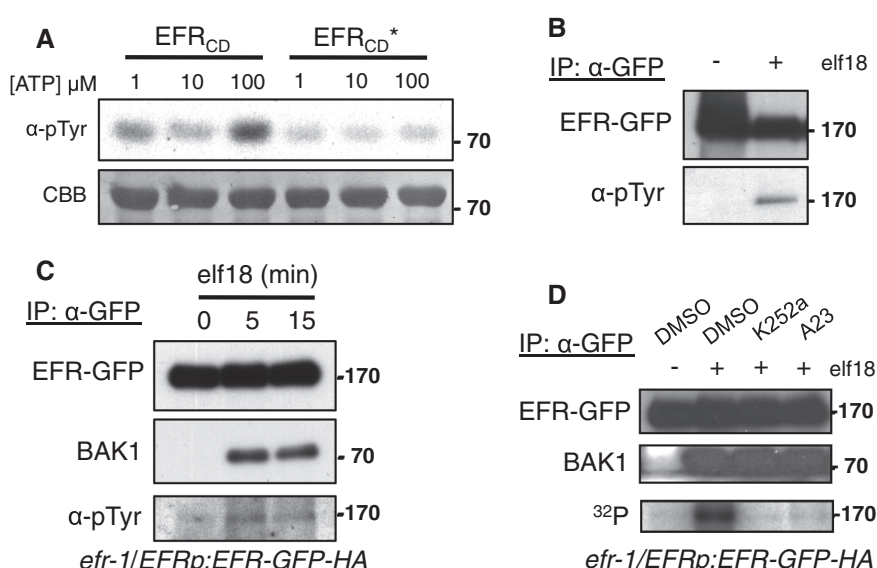


Fig. 3. Y836 is important for EFR function and Tyr phosphorylation. The expression of EFR^{Y836F} compromises elf18-triggered responses in *N. benthamiana* (A) or *Arabidopsis* *efr-1* mutant plants (B to F). (A and B) ROS production presented as total photon counts during 40 min after treatment with 100 nM elf18. Values are means $\pm$ SE, $n = 12$. RLU, relative luminescence units. (C) *Arabidopsis* EFR-GFP mesophyll protoplasts were transfected with a plasmid expressing *35S::BIK1-HA* and treated with water (–) or 100 nM elf18 (+) for 10 min. BIK1 phosphorylation is detected by a mobility shift of the BIK1-HA band. (D) Phosphorylation of MAPKs after treatment with water (–) or 100 nM elf18 (+) for 10 min. (E) Growth of syringe-infiltrated *Pto* DC3000 [10^5 colony-forming units (CFU)/ml] in leaves pretreated with water (mock) or 1 μ M elf18 for 24 hours. Bacterial numbers were determined 2 days after inoculation. Values are mean $\pm$ SE, $n = 4$ [one-way analysis of variance (ANOVA); $P < 0.05$]. (F) Growth of surface-inoculated *Pto* DC3000 $\Delta avrPto\Delta avrPtoB$ (10^7 CFU/ml), determined 3 days after inoculation.

Values are means $\pm$ SE, $n = 4$. (G) Recombinant MBP-EFR was incubated with [32 P]-ATP. EFR*, kinase-dead version (D849N). In vitro phosphorylation is revealed by autoradiography. CD, cytoplasmic domain. CBB, Coomassie brilliant blue. (H) Activation of EFR-GFP immunoprecipitated from *Arabidopsis* seedlings treated with water (–) or 100 nM elf18 (+) for 10 min. Immunoprecipitated EFR-GFP was incubated with [32 P]-ATP. In vitro phosphorylation is revealed by autoradiography. (I) Mean $\pm$ SE of densitometry measurements from three independent replicates of the assay shown in (H). (J) Tyr phosphorylation on EFR-GFP immunoprecipitated from *Arabidopsis* seedlings after treatment with water (–) or 100 nM elf18 (+) for 10 min. Immunoblots were analyzed with anti-pTyr, anti-GFP or anti-BAK1. (K) Average of densitometry measurements from three independent replicates of the assay shown in (J). Asterisks indicate mean values significantly different from EFR wild type (Student's *t* test; * $P < 0.05$; *** $P < 0.001$). Experiments were performed three times with similar results.

FLS2 (12). The EFR cytoplasmic domain was able to phosphorylate on Tyr residues in vitro (Fig. 2A and fig. S2). This phosphorylation was dependent on EFR catalytic activity (Fig. 2A), demonstrating that EFR undergoes autophosphorylation on Tyr residues.

Immunoprecipitation (IP) of EFR transiently expressed in *Nicotiana benthamiana* showed that EFR is phosphorylated on Tyr residues in planta specifically after elicitation with elf18 (Fig. 2B).

Similarly, elf18 induced EFR Tyr phosphorylation in *Arabidopsis* (Fig. 2C). Other Tyr-phosphorylated proteins coimmunoprecipitated with EFR, including a band of about 70 kD, which likely corresponds to BAK1 or related proteins (fig. S3). These results demonstrate that EFR undergoes Tyr phosphorylation in vivo in a ligand-dependent manner.

We next tested the role of Tyr phosphorylation in the elf18-induced activation of EFR. To measure EFR phosphorylation, we performed an in vitro ki-

nase assay on EFR immunoprecipitated from *Arabidopsis* seedlings that were or were not pretreated with A23. In this assay, EFR phosphorylation is only detectable after elf18 perception, and this was blocked by A23 treatment (Fig. 2D). The inhibition of EFR phosphorylation was also observed after addition of A23 during the in vitro kinase assay (fig. S4). Together, these results reveal that elf18-dependent Tyr phosphorylation of EFR is essential for the activation of EFR.

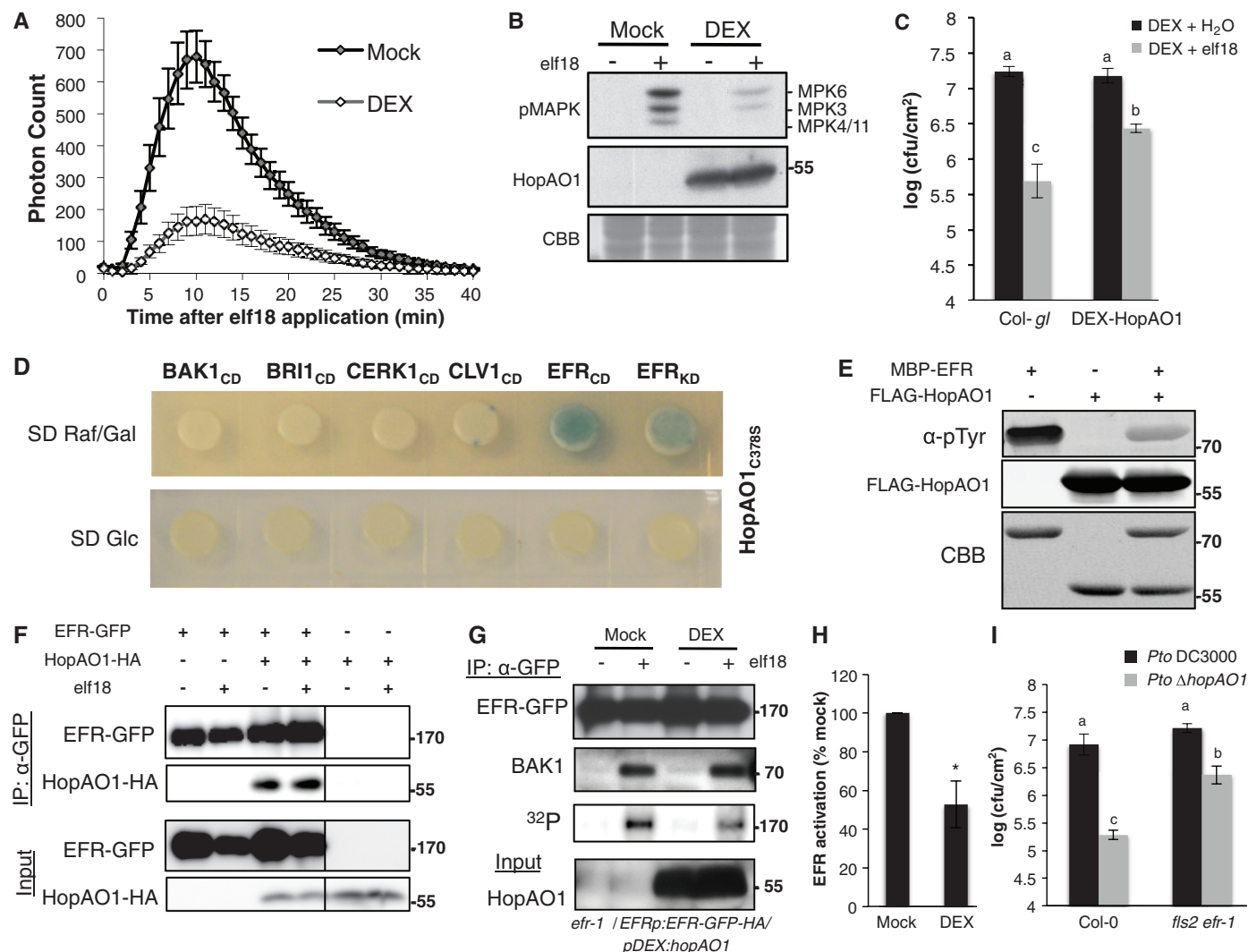


Fig. 4. HopAO1 targets EFR Tyr phosphorylation. Dexamethasone (DEX)-induced expression of HopAO1 in *Arabidopsis* compromises several elf18-induced responses. **(A)** ROS burst measured after a 16 hours' pretreatment with a solvent control (mock) or 30 μ M DEX and subsequent treatment with 100 nM elf18. Values are means $\pm$ SE, $n = 12$. **(B)** MAPK activation after treatment with water (–) or 100 nM elf18 (+) for 10 min. Seedlings were pretreated for 16 hours with a solvent control (mock) or 30 μ M DEX. CBB, Coomassie brilliant blue. **(C)** Growth of syringe-infiltrated *Pto* DC3000 (10^5 CFU/ml) in leaves pretreated with 5 μ M DEX for 24 hours, and then treated with water (mock) or 1 μ M elf18 for 24 hours. Bacterial numbers were determined 2 days after inoculation. Values are means $\pm$ SE, $n = 4$ (one-way ANOVA; $P < 0.05$). **(D)** Yeast-two-hybrid assays to determine the interaction of HopAO1_{C378S} with EFR, BAK1, BRI1, CERK1, and CLV1. CD, cytoplasmic domain; KD, kinase domain. Blue colonies on SD Raf/Gal induction plate indicate positive interaction. **(E)** HopAO1 Tyr phosphatase assay in vitro. Phosphorylated recombinant MBP-EFR was incubated with recombinant FLAG-

HopAO1. Immunoblots were analyzed with anti-pTyr or anti-FLAG. CBB, Coomassie brilliant blue. **(F)** Coimmunoprecipitation of EFR-GFP and HopAO1-HA in *N. benthamiana* after treatment with water (–) or 100 nM elf18 (+) for 10 min. Immunoblots were analyzed with anti-GFP or anti-HA. **(G)** EFR activation in *Arabidopsis* seedlings after a 16 hours' pretreatment with a solvent control (mock) or 30 μ M DEX and subsequent treatment with water (–) or 100 nM elf18 (+) for 10 min. Immunoprecipitated EFR-GFP was incubated with [³²P]γ-ATP. Immunoblots were analyzed with anti-GFP, anti-BAK1, and anti-HopAO1. In vitro phosphorylation is revealed by autoradiography. **(H)** Mean $\pm$ SE of densitometry measurements from three independent biological replicates of the assay shown in (G). Asterisk indicates mean values significantly different from mock (Student's *t* test; * $P < 0.05$). **(I)** Growth of syringe-inoculated *Pto* DC3000 or *Pto* DC3000 ΔhopAO1 (5×10^4 CFU/ml), determined 3 days after inoculation. Values are means $\pm$ SE, $n = 4$ (one-way ANOVA; $P < 0.05$). Experiments were performed three times with similar results.

Previously characterized Tyr residues in BRI1 and BAK1 were identified through targeted mutagenesis (6, 7). Therefore, we carried out site-directed mutagenesis to substitute individually each of the 11 Tyr residues present in the EFR cytoplasmic domain with a Phe (F) residue, which lacks the phosphorylatable hydroxyl group. We tested functionality of these variants by expressing them transiently in *N. benthamiana*, which lacks endogenous EFR but otherwise can activate *elf18*-induced immune responses upon EFR expression (13). All variants accumulated to levels similar to that of wild-type EFR (fig. S5). Of the 11 EFR variants tested, only EFR^{Y836F} was fully compromised in mounting an *elf18*-induced ROS burst (Fig. 3A and fig. S5). Stable transgenic expression of EFR^{Y836F} in *Arabidopsis* did not complement the null *efr-1* mutant phenotype. Lines expressing EFR^{Y836F} were compromised in *elf18*-triggered ROS burst generation, activation of BIK1, and mitogen-activated protein kinases (MAPKs) (Fig. 3, B to D). The EFR^{Y836F} line was also less resistant to the phytopathogenic bacterium *Pseudomonas syringae* pv. *tomato* (Pto) DC3000 in a disease protection assay induced by *elf18* pretreatment (Fig. 3E). Additionally, the EFR^{Y836F} line was more susceptible to surface inoculation with the weakly virulent strain Pto DC3000 Δ avrPto Δ avrPtoB (Fig. 3F). These results confirm the importance of EFR^{Y836} in *elf18*-triggered immunity.

The Y836 residue is located in the kinase subdomain VIa of EFR, is phosphorylated upon *elf18* perception in vivo (fig. S6), and is conserved in PRRs and other receptor kinases (fig. S7). EFR^{Y836F} properly accumulates, localizes, and associates with BAK1 in a ligand-dependent manner (Fig. 3 and figs. S5C and S8). EFR^{Y836F} is fully catalytic active and has a kinase activity similar to that of wild-type EFR in vitro (Fig. 3G). The Y839F mutation reduced overall *elf18*-induced phosphorylation of EFR (Fig. 3, H and I) and Tyr phosphorylation of EFR in vivo (Fig. 3, J and K). Thus, Y836 is a major Tyr phosphorylation site of EFR or is required for the phosphorylation of other EFR Tyr residues. Other Tyr residues, such as Y897, also contribute to the overall ligand-induced Tyr phosphorylation of EFR (fig. S9) but are not as important for downstream signaling (fig. S5). These results provide a mechanistic link between ligand-induced activation of EFR, Tyr phosphorylation (Y836) of the receptor, and the initiation of downstream immune signaling.

Many bacterial pathogens inject effector proteins into the host cell via the type III secretion system to suppress immune processes and components (14). One such effector conserved in several *P. syringae* pathovars is HopAO1 (formerly known as HopPtoD2), a protein tyrosine phosphatase (PTP) that contributes to virulence (15–18). Consistent with previous results (15, 17), we show that inducible expression of HopAO1 in *Arabidopsis* leads to impaired early immune responses, such as *elf18*-triggered ROS burst and MAPK activation (Fig. 4, A and B), as well as *elf18*-induced resistance to Pto DC3000 (Fig. 4C). Similarly, HopAO1 inhibited *flg22*-induced ROS burst (fig.

S10). Despite its virulence activity and appreciable contribution to overall virulence of Pto DC3000, the plant target(s) for HopAO1 is still unknown.

We initially identified the kinase domain of FLS2 as one of the interactors of HopAO1 in a yeast two-hybrid (Y2H) screen using an *Arabidopsis* cDNA library (fig. S10). A catalytically inactive form of HopAO1 (HopAO1^{C378S}) (17) was used to stabilize potential interactions that otherwise may be transient. Targeted Y2H experiments showed that HopAO1^{C378S} directly interacts with both the kinase and cytoplasmic domains of EFR and FLS2 (Fig. 4D and fig. S10). HopAO1^{C378S} did not interact with the cytoplasmic domain of BAK1, the chitin receptor CERK1, or the receptor kinases BRI1 and CLV1 involved in growth and development, illustrating the specificity of the interactions (Fig. 4D). Consistent with direct interaction, HopAO1 led to a reduced Tyr phosphorylation on EFR in vitro (Fig. 4E). We confirmed this interaction between catalytically active HopAO1 and EFR in planta after transient expression of both proteins in *N. benthamiana* (Fig. 4F). HopAO1 did not interfere with the subcellular localization of EFR (fig. S8) or with its ligand-dependent association with BAK1 (Fig. 4G). These observations indicate that EFR (and potentially FLS2) is a plant target of HopAO1.

Next, we tested whether the interaction of EFR with HopAO1 affects its ligand-induced phosphorylation. Indeed, HopAO1 expression led to a ~50% reduction in the phosphorylation of EFR upon *elf18* treatment (Fig. 4, G and H). This reduction was partially dependent on HopAO1 catalytic activity (fig. S11). We could still observe about 20% inhibition of EFR activity by catalytically inactive HopAO1^{C378S}. This indicates that the physical interaction of both proteins itself may inhibit EFR phosphorylation. Although we cannot study FLS2 phosphorylation owing to its very low kinase activity, the fact that HopAO1 also interacts with FLS2 and inhibits *flg22*-induced responses (fig. S10) (17) suggests that HopAO1 would also affect its Tyr phosphorylation. In summary, our results indicate that one of the virulence functions of HopAO1 is to target the Tyr phosphorylation of PRRs, such as EFR, thereby inhibiting their ligand-induced activation and downstream immune signaling. Consistent with FLS2 and EFR being important virulence targets for HopAO1 during infection, the virulence defect of Pto DC3000 lacking HopAO1 (Pto DC3000 Δ hopAO1) was alleviated in *fls2 efr-1* double-mutant plants (Fig. 4I).

Our results demonstrate that Tyr phosphorylation drives activation of plant PRRs upon ligand binding. Consistent with its importance in triggering immune responses, this specific posttranslational modification is targeted by the *Pseudomonas* type III-secreted effector HopAO1, which is an active tyrosine phosphatase (15, 16). Animal bacterial pathogens deploy effector proteins that target Tyr phosphorylation, e.g., *Yersinia* YopH or *Salmonella* SptP (19), but none of them have been found to target PRRs or associated kinases. The role of Tyr phosphorylation in PRR activation, the important role of HopAO1 in *Pseudomonas* virulence, and

the presence of PTP domains in type III-secreted effectors from several bacterial pathogens (15, 16, 18) illustrate that Tyr phosphorylation is a conserved mechanism important for antibacterial immunity across kingdoms.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S11
Table S1
References (20–24)

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A Non–Cell Autonomous Role of *E(z)* to Prevent Germ Cells from Turning on a Somatic Cell Marker

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In many metazoans, germ cells are separated from somatic lineages early in development and maintain their identity throughout life. Here, we show that a Polycomb group (PcG) component, Enhancer of Zeste [*E(z)*], a histone transferase that generates trimethylation at lysine 27 of histone H3, maintains germline identity in *Drosophila* adult testes. We find excessive early-stage somatic gonadal cells in *E(z)* mutant testes, which originate from both overproliferative cyst stem cells and germ cells turning on an early-stage somatic cell marker. Using complementary lineage-tracing experiments in *E(z)* mutant testes, a portion of excessive early-stage somatic gonadal cells are found to originate from early-stage germ cells, including germline stem cells. Moreover, knocking down *E(z)* specifically in somatic cells caused this change, which suggests a non–cell autonomous role of *E(z)* to antagonize somatic identity in germ cells.

The dichotomy of germ line and soma represents the earliest lineage specification among many metazoan organisms (1–4). *Drosophila* has provided a paradigmatic system to study germ cell establishment and maintenance. During *Drosophila* development, primordial germ cells are specified in early embryogenesis, which requires suppression of somatic gene expression (5–7). Specified primordial germ cells migrate

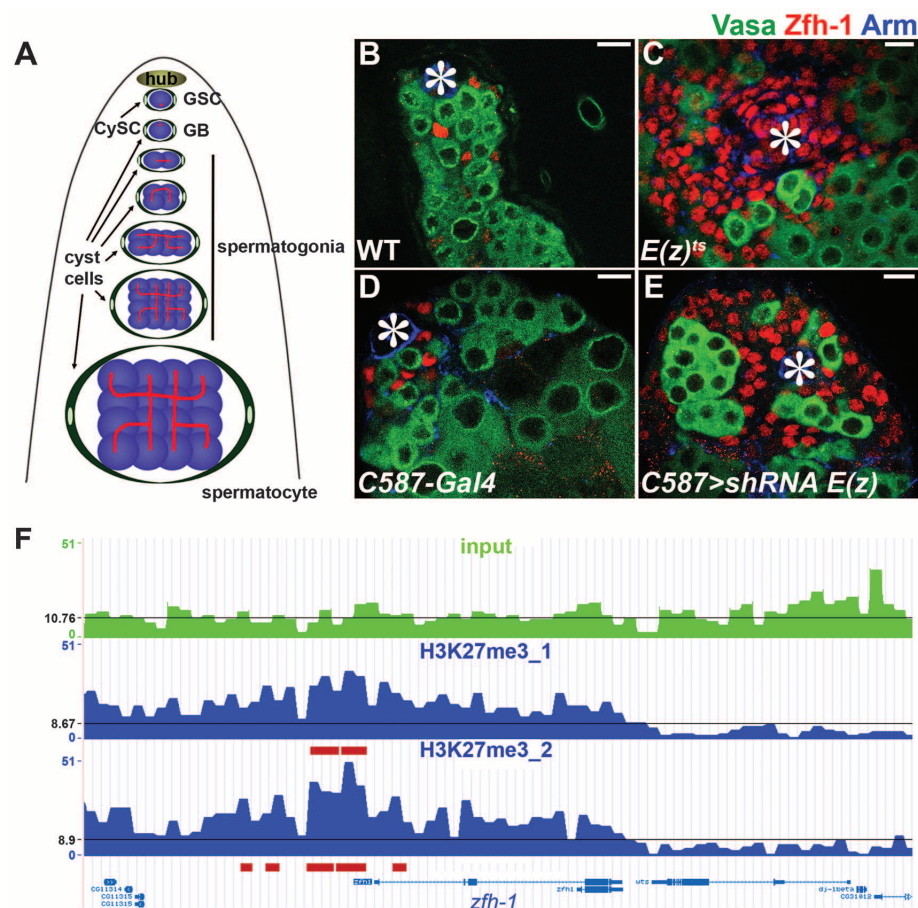
and interact with mesodermal somatic cells to establish germline stem cells (GSCs) (8, 9). In adult testes, GSCs interact with two populations of somatic gonadal cells: postmitotic hub cells and cyst stem cells (CySCs) (10, 11) (Fig. 1A). Because germline-to-soma conversion has only been reported at the embryonic stage, which leads to germ cell death (12), it is unclear whether germ cells retain plasticity in adult gonads.

Polycomb group (PcG) proteins maintain somatic cell fate decisions, and mutations in *PcG* genes often lead to transdifferentiation through which one somatic cell type adapts a different somatic identity (13). Here, we investigate a PcG gene, *Enhancer of Zeste* [*E(z)*], in *Drosophila* adult testes. *E(z)* encodes a histone transferase that generates trimethylation at lysine 27 of histone H3 (H3K27me3) (14). To inactivate *E(z)* after a shift in temperature, a null allele of *E(z)*, *E(z)⁷³¹*, was used in trans to a temperature-sensitive (ts) allele, *E(z)⁶¹* (15). We found that the H3K27me3 mark was not detectable by immunostaining in *E(z)⁶¹/E(z)⁷³¹* testes after a temperature shift (fig. S1, A to F). We then examined the expression of zinc-finger homeodomain protein 1 (*Zfh-1*), a marker for CySCs and early-stage cyst cells (16) (Fig. 1, B and D). The overall *Zfh-1*–positive CySCs and early cyst cells ranged from 20 to 59 per wild-type (WT) testis, but their numbers increased markedly in ~20% of *E(z)⁶¹/E(z)⁷³¹* testes under the same condition (Fig. 1C and fig. S2A).

To determine in which cell type loss of *E(z)* function leads to excessive *Zfh-1*–positive cells,

Fig. 1. Loss of function of *E(z)* leads to excessive *Zfh-1*–expressing cells in adult testes. (A)

A schematic diagram of *Drosophila* adult testis. The spherical spectrosomes (red dots) are present in GSCs and gonialblasts (the daughter cell of GSC, which undergoes differentiation), whereas branched fusomes (red lines) appear when germ cells undergo differentiation into both spermatogonia and spermatocytes. Germ cells (blue) are encapsulated by cyst cells (green). (B to E) Immunostaining using antibody against *Zfh-1* (α -*Zfh-1*) (red labeling of early cyst cells, including CySCs), α -*Vasa* (green labeling of all germ cells), and α -*Armadillo* (Arm, blue labeling of hub cells, as indicated by asterisks) in testes from (B) WT and (C) *E(z)⁶¹/E(z)⁷³¹* males after shifting temperature from 25° to 29°C for 28 days as adult flies and (D) *C587-Gal4* control and (E) *C587-Gal4; UAS-shRNA E(z)* males after shifting temperature from 18° to 29°C for 21 days as adult flies. Scale bars, 10 μ m. (F) A genome browser snapshot at the *zfh-1* gene locus of H3K27me3 ChIP-seq data using *nos>shRNA E(z)* testes, where H3K27me3 is only detected in somatic cells (fig. S1, J to L). H3K27me3 is enriched at the *zfh-1* gene region in two H3K27me3 ChIP-seq replicates (marked by red lines) but not in the input. The read density was binned into a 1-kilobase (kb) window, and the black line indicates the average read density at chromosome 3R.



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we depleted (knocked down) *E(z)* using short hairpin RNA driven by an upstream activation sequence (*UAS-shRNA*) (17), in combination

with different cell type-specific Gal4 drivers (18). Subsequent immunostaining experiments demonstrated that knockdown of *E(z)* in cyst cells [by

C587-Gal4 (19)] or germ cells [by *nanos/nos-Gal4* (20)] resulted in a cell type-specific loss of H3K27me3 (fig. S1, G to L). We found a substantial

Fig. 2. *E(z)* is required in somatic cells to prevent accumulation of excessive *Zfh-1*-expressing cells and cells with both germline and somatic lineage markers. (A to C) EdU (green) and *Zfh-1* (red) double-positive cells representing dividing somatic cells are detected in CySCs adjacent to hub in (A) *C587-Gal4* control but far from hub in (B) *C587>shRNA E(z)* (short hairpin RNA) testes (21 days at 29°C) and (C) *E(z)⁶¹/E(z)⁷³¹* testes (28 days at 29°C), α -Vasa (blue). Arrowheads indicate dividing CySCs next to hub; arrows indicate dividing cyst cells away from hub. (D to F) Cells with Vasa [(D) and green in (F)] and *Zfh-1* [(E) and red in (F)] signals in a cluster of cells in *C587>shRNA E(z)* testes (21 days at 29°C). (Insets) An enlarged cell indicated by yellow arrowheads. (G to I) Cells with both Vasa [(G) and green in (I)] and *Zfh-1* [(H) and red in (I)] signals in a cluster of cells in *E(z)⁶¹/E(z)⁷³¹* mutant testes (28 days at 29°C), α -Arm (blue). Hub, asterisks. Scale bars, 10 μ m.

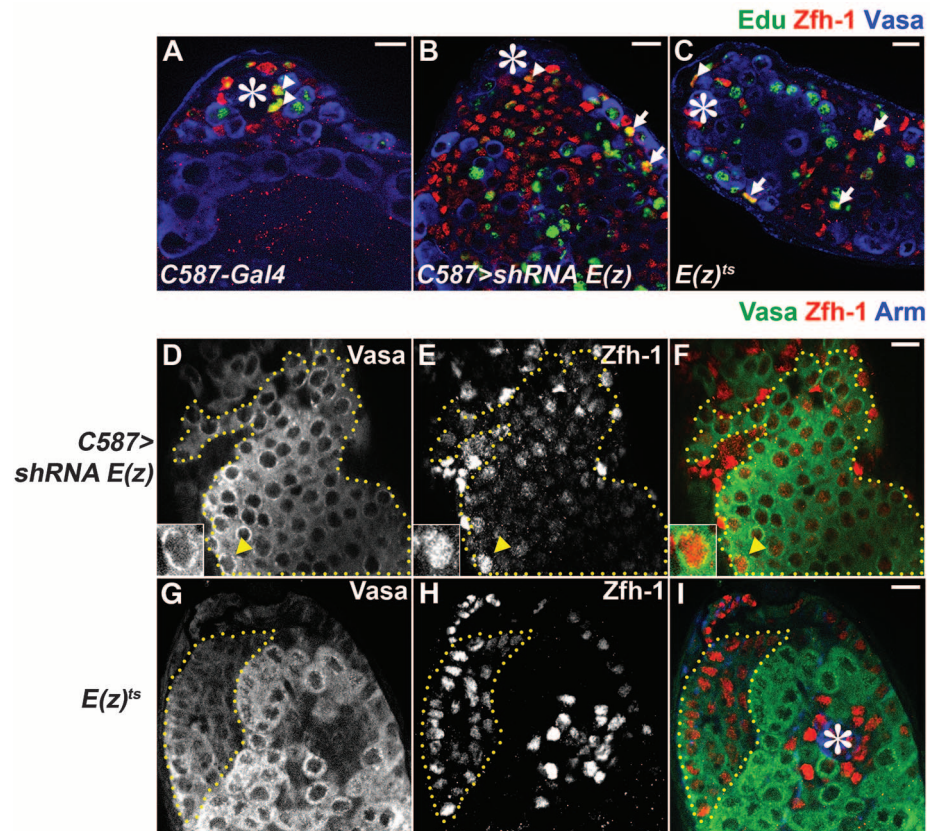
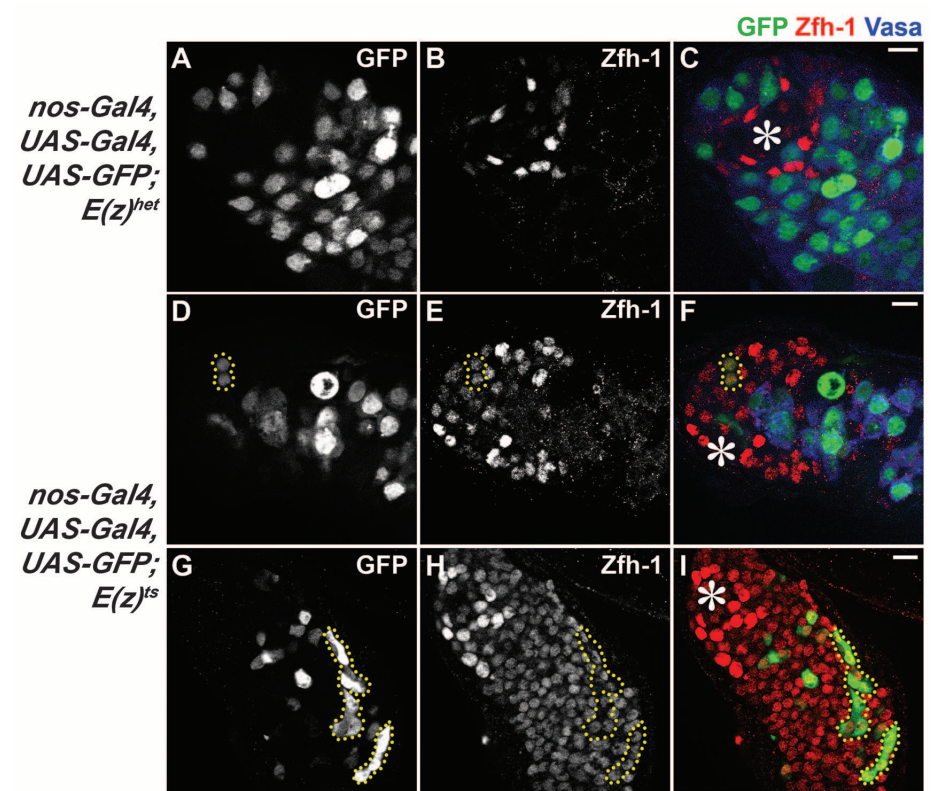


Fig. 3. Some *Zfh-1*-positive cells are derived from germline lineage. Lineage tracing with permanently labeled germline cells using autoregulatory Gal4-controlled gene expression. The *nanos-Gal4>UAS-Gal4>UAS-GFP* transgenes in (A to C) *E(z)* heterozygous (28 days at 29°C) and (D to I) *E(z)⁶¹/E(z)⁷³¹* testes (28 days at 29°C). Detection of GFP and *Zfh-1* double-positive cells (yellow dotted outlines) suggests that some *Zfh-1*-expressing cells are derived from germline lineage. Hub, asterisks. Scale bars, 10 μ m.



overpopulation of *Zfh-1*-positive cells when *UAS-shRNA E(z)* was driven by either the *C587-Gal4* driver (Fig. 1E and fig. S2B) or another cyst cell-specific *eya-Gal4* driver (21) (fig. S2B). By contrast, the same *UAS-shRNA E(z)*, when driven by either the germline-specific *nos-Gal4* driver (22) or a hub-specific *unpaired-Gal4* (*upd-Gal4*) driver, did not recapitulate these phenotypes (fig. S2B). A similar phenotype was also detected when another PcG component, *Su(z)12*, was knocked down in *C587>shRNA Su(z)12* testes (fig. S2B). Together, these results suggest that *E(z)* is required in CySCs and cyst cells to prevent the accumulation of excessive *Zfh-1*-positive cells in adult testes. *Zfh-1* is a known downstream target of the Janus kinase–signal transducers and activators of transcription (JAK-STAT) signaling pathway (18, 21). However, we found no difference in JAK-STAT activity, as represented by the Stat92E immunostaining pattern (21, 23), in control testes compared with *E(z)* mutant testes [both *E(z)^{ts}* and *C587>shRNA E(z)* (fig. S3)], which suggests that the ectopic expression of *Zfh-1* in *E(z)* mutant testes is not directly caused by hyperactive JAK-STAT signaling. Consequently, we speculated that *zfh-1* might be a direct target of PcG in somatic gonadal cells. To test this, we performed an H3K27me3 chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) experiment (fig. S4) using *nos>shRNA E(z)* testes

in which H3K27me3 was only detected in somatic cells (fig. S1, J to L). Enrichment of the H3K27me3 mark was found at the *zfh-1* gene locus (Fig. 1F), which suggests that *zfh-1* is, in fact, directly bound and repressed by PcG in somatic gonadal cells.

To investigate whether overpopulated *Zfh-1*-positive cells in *E(z)* mutant testes are derived from ectopically dividing CySCs, a pulse 5-ethynyl-2'-deoxyuridine (EdU) incorporation was performed to label S-phase cells (24). In WT adult testes, CySCs, but not cyst cells (Fig. 1A), are mitotically active (25). Accordingly, EdU signals were detected in *Zfh-1*-positive CySCs next to the hub in the control testes (arrowheads in Fig. 2A). However, EdU and *Zfh-1* double-positive somatic cells were detected away from the hub in *E(z)* mutant testes (arrows in Fig. 2, B and C). In addition, all *Zfh-1*-positive cells in the *C587-Gal4* control testes expressed an early somatic cell marker, Tj (fig. S5, A to C), whereas most of the overpopulated *Zfh-1*-positive cells in *C587>shRNA E(z)* (fig. S5, D to F) and *E(z)⁶¹/E(z)⁷³¹* (fig. S5, G to I) testes lacked Tj expression. Furthermore, EdU and Vasa double-positive germ cells were detected in the middle, or toward the basal region, of both *C587>shRNA E(z)* (31.7%, $n = 63$) (fig. S6, A to C and H) and *E(z)⁶¹/E(z)⁷³¹* (6.1%, $n = 33$) (fig. S6, D to F and J) testes, whereas they were only detectable at the tip of the control testes

[$n = 44$ (fig. S6G); $n = 32$ (fig. S6I)]. These results were further confirmed using a mitosis-specific marker, phosphorylated serine 10 of histone 3 (phH3) (fig. S6, K to N). We also found several unique cellular features of these ectopically dividing germ cells. First, they divided like GSCs or gonialblasts (Fig. 1A), as shown by single germ cells labeled by EdU (white arrowheads in fig. S6, C and F) and germ cells with round spectrosomes (yellow arrows in fig. S7, C and D), consistent with previous studies showing that sustained *Zfh-1* expression in cyst cells leads to excessive GSC self-renewal away from the niche (21). However, different from the previous report (21), these ectopically dividing germ cells failed to intermingle with *Zfh-1*-expressing cells (yellow arrowheads in fig. S6, B, C, E, and F). It is possible that lack of Tj expression in those overpopulated *Zfh-1*-positive cells in *E(z)* mutant testes abolished their proper association with germ cells (26). Second, we found that some ectopically dividing GSC-like cells turned on *Zfh-1* expression in *E(z)* mutant testes, which led to cells with both cytoplasmic Vasa staining and nuclear *Zfh-1* staining (Fig. 2, D to I). The Vasa and *Zfh-1* double-positive cells could be detected in ectopically dividing germ cell clusters that lacked association with cyst cells in *E(z)* mutant testes (Fig. 2, D to I). Notably, although the subcellular localization of Vasa and *Zfh-1* in these double-positive cells was normal, their immunostaining signals were usually weaker than those of cells with a single signal (Fig. 2, G to I), which suggests some type of intermediate cellular state.

The observation of Vasa and *Zfh-1* double-positive cells raised a possibility of mixed cell fate. To test this idea, we used a lineage-tracing regimen to permanently label germ cells with the *nos-Gal4>UAS-Gal4>UAS-GFP* transgene and green fluorescent protein (GFP) combination, which irreversibly labels all GSCs and their derivatives with GFP (9). Indeed, we detected GFP and *Zfh-1* double-positive cells in *nos-Gal4>UAS-Gal4>UAS-GFP;E(z)⁶¹/E(z)⁷³¹* testes (18.9%, $n = 53$) (Fig. 3, D to I) after temperature shift, but not in control testes ($n = 47$) (Fig. 3, A to C), which suggests ectopic expression of *Zfh-1* in germ cells upon inactivation of *E(z)*. We also used the G-TRACE lineage tracing method combining *UAS-FLP*, *UAS-RFP* (with red fluorescent protein), and *ubi-FRT-STOP-FRT-GFP* transgenes (27) with the *nos-Gal4* driver, which labels all germline lineage cells with GFP and early-stage germ cells with RFP. With G-TRACE, we found *Zfh-1* and GFP double-positive cells in *E(z)⁶¹/E(z)⁷³¹* testes (17.1%, $n = 76$) (fig. S8, E to H) but not in control testes ($n = 95$) (fig. S8, A to D). We also found *Zfh-1* and RFP double-positive cells in *E(z)⁶¹/E(z)⁷³¹* testes (9.2%, $n = 76$) (arrowheads in fig. S8, E to H) but not in control testes ($n = 95$) (fig. S8, A to D). All *Zfh-1* and RFP double-positive cells in *E(z)⁶¹/E(z)⁷³¹* testes were also labeled by GFP, which suggests that they represent a subset of germline lineage cells that transiently express both lineage markers, similar to the Vasa

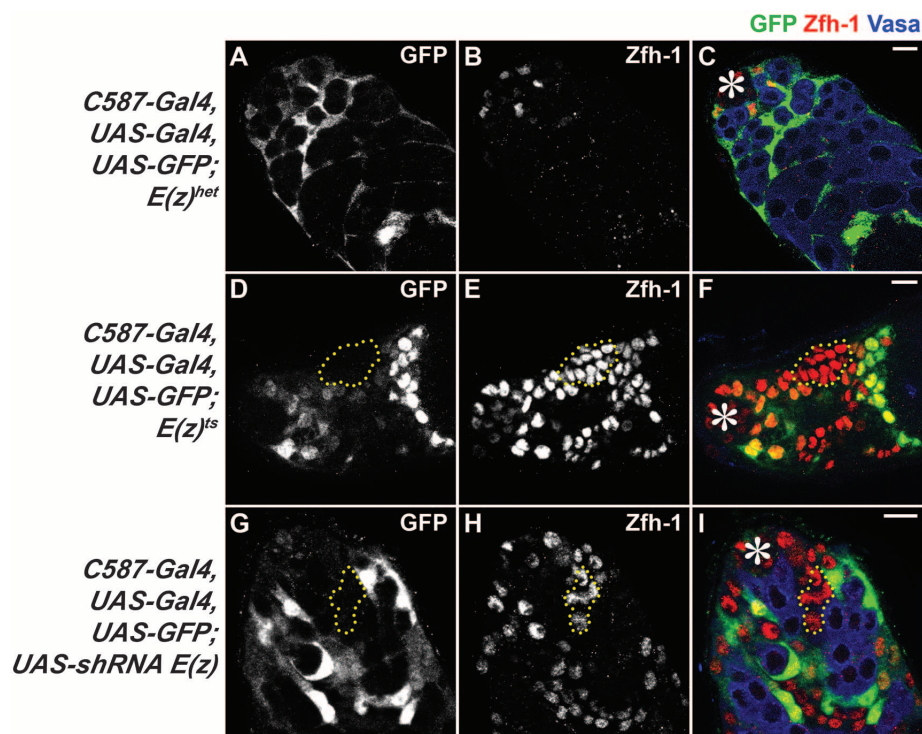


Fig. 4. Some *Zfh-1*-positive cells are not derived from somatic gonadal cell lineage. Lineage tracing with permanently labeled somatic-lineage cells. The *C587-Gal4>UAS-Gal4>UAS-GFP* transgene in (A to C) *E(z)* heterozygous (28 days at 29°C); (D to F) *E(z)⁶¹/E(z)⁷³¹* (28 days at 29°C); and (G to I) *UAS-shRNA E(z)* testes (21 days at 29°C). Hub, asterisks. Scale bars, 10 μ m. Detection of GFP-negative and *Zfh-1*-positive cells (outlined by yellow dotted lines) suggests that these cells are not derived from somatic cell lineage.

and Zfh-1 double-positive cells shown previously (Fig. 2, F and I). Therefore, using two different lineage tracing methods, we found that germ cells turned on a key somatic cell-specific transcription factor, Zfh-1, when E(z) was inactivated in somatic gonadal cells. Notably, the nuclear morphology of Zfh-1-positive cells originated from germline lineage was indistinguishable from that of CySC lineage-derived Zfh-1-positive cells (Fig. 3, E and H, and fig. S8G). Furthermore, no spectrosome or fusome structure could be detected in those overpopulated Zfh-1-positive cells (fig. S7, C and D), which suggests that the germline-derived Zfh-1-positive cells eventually lose germ cell features. Lineage-tracing experiments were also performed using *bam-Gal4*, which drives GFP expression in more differentiated germ cells from four-cell spermatogonia to later stages (28). In *bam-Gal4>UAS-Gal4>UAS-GFP; E(z)⁶¹/E(z)⁷³¹* testes, no Zfh-1-positive cells were found to be labeled by GFP ($n = 76$) (fig. S9), which suggests that only GSCs, gonialblasts, and/or two-cell spermatogonia are capable of turning on Zfh-1 expression.

In a complementary lineage-tracing experiment, we used a somatic driver to permanently label all CySC lineage cells. In *C587-Gal4>UAS-Gal4>UAS-GFP* testes, no GFP and Vasa double-positive cells could be detected in either *E(z)⁶¹/E(z)⁷³¹* ($n = 32$) or *C587>shRNA E(z)* ($n = 45$) testes, which suggests that somatic lineage cells did not express the germline marker. However, we did find that some Zfh-1-positive cells lacked the GFP marker in both *E(z)⁶¹/E(z)⁷³¹* (50.0%, $n = 32$) (Fig. 4, D to F, yellow outline) and *C587>shRNA E(z)* (68.9%, $n = 45$) (Fig. 4, G to I, yellow outline) testes, which indicates that some Zfh-1-expressing cells were not derived from the CySC lineage. By contrast, all Zfh-1-positive cells showed GFP labeling in the controls ($n = 26$) (Fig. 4, A to C). Consistent with these lineage tracing results, our H3K27me3 ChIP-seq data showed lack of H3K27me3 at the germline gene loci in somatic gonadal cells (fig. S10), which suggests that germline-specific genes are not direct targets of PcG in somatic cells. Taken together, these results show that germline lineage cells also contribute to Zfh-1-expressing cells in *E(z)* mutant testes.

Because these results suggest that E(z) has a non-cell autonomous role, we hypothesize that E(z) regulates signaling pathway(s) for proper communication between germ cells and somatic cells. Our H3K27me3 ChIP-seq data in somatic gonadal cells showed enrichment of H3K27me3 at several signaling pathways, including Wnt (fig. S11) and epidermal growth factor (EGF) (fig. S12A) pathway genes' loci. Furthermore, we found that removal of one copy of the EGF receptor gene, *Egfr*, suppresses the *E(z)⁶¹/E(z)⁷³¹* phenotype (fig. S12B), which suggests that *Egfr* is a direct target of PcG in somatic gonadal cells and may connect E(z) activity in soma with the observed germline defects.

In summary, we demonstrate that the PcG gene *E(z)* is required in somatic gonadal cells to prevent ectopic early-stage somatic and germ cell proliferation. Moreover, E(z) is required in somatic

cells to prevent germ cells from expressing a somatic cell marker (fig. S13). In *Caenorhabditis elegans*, it has been reported that the germ line can convert to specific neurons upon ectopic germline expression of neuron-specific transcription factors and loss of a key histone chaperone, LIN-53 (29). Removal of *mes-2*, the *C. elegans* homolog of *E(z)*, also makes germ cells more susceptible to conversion to somatic cell types (30). However, it is unclear in which cell type LIN-53 and MES-2 act to maintain germline identity, because its function is ubiquitously compromised in those experiments. Here, using cell type-specific knockdown experiments, we demonstrate that the germline-to-soma change relies on the activity of E(z) in somatic gonadal cells, which suggests that cell fate maintenance depends on the microenvironment or niche where the cells reside.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S13
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The *Drosophila* Circadian Clock Is a Variably Coupled Network of Multiple Peptidergic Units

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Daily rhythms in behavior emerge from networks of neurons that express molecular clocks. *Drosophila*'s clock neuron network consists of a diversity of cell types, yet is modeled as two hierarchically organized groups, one of which serves as a master pacemaker. Here, we establish that the fly's clock neuron network consists of multiple units of independent neuronal oscillators, each unified by its neuropeptide transmitter and mode of coupling to other units. Our work reveals that the circadian clock neuron network is not orchestrated by a small group of master pacemakers but rather consists of multiple independent oscillators, each of which drives rhythms in activity.

Molecular clocks drive circadian rhythms in animals (1). Most circadian rhythms follow from clocks located in small is-

lands of brain tissue (2), and connections within networks of clock neurons produce a robustness in circadian timekeeping uncharacteristic of rhythms driven by isolated neurons or non-neuronal clocks (3, 4). Here, we study the clock neuron network of *Drosophila*, which is similar to yet simpler than that of mammals (5), to learn how

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networks of clock neurons produce circadian rhythms.

The *Drosophila* brain contains ~150 clock neurons, of which 11 bilateral pairs of lateral neurons are necessary and sufficient for the insect's normal activity rhythms (6, 7) (fig. S1). Current models suggest that this network is organized into two coupled oscillators: the pigment-dispersing factor (PDF) expressing lateral neurons that control the morning peak of activity and the remaining lateral neurons that control the evening peak of activity (6, 7) (fig. S1). The dual-oscillator model predicts

that the PDF-positive neurons serve as master pacemakers that reset the PDF-negative neurons daily, thereby dictating the pace of behavioral rhythms in the absence of environmental time cues (8). We tested this prediction by introducing various clock speed discrepancies between the PDF-positive and -negative clock neurons (see supplementary materials and methods).

The intrinsic speed of the molecular clock can be manipulated through the activity of the kinases Doubletime (DBT) and Shaggy (SGG) (9, 10) (Fig. 1A, fig. S2, and table S1). Manipulating these kinases only in the PDF-positive clock neurons resulted in a coherent change in clock speed in these neurons (fig. S3), thereby creating clock speed discrepancies between PDF-positive and -negative neurons. When these discrepancies were small, activity rhythms were strong and coherent with periodicities determined by the speed of the PDF neurons (Fig. 1B, figs. S4 and S5, and table S2). When speed discrepancies were larger, flies

displayed variable free-running periods, reduced rhythm amplitudes, and a higher incidence of arrhythmicity (Fig. 1B, figs. S4 and S5, and table S2). Flies with large discrepancies often displayed two periodicities simultaneously, one corresponding to the period of the PDF-positive neurons and the other to that of the PDF-negative neurons (Fig. 1B and figs. S4 and S5). In flies lacking PDF receptors (PDFRs), the speed of PDF neurons had no influence over activity rhythms (Fig. 1C, fig. S6, and table S3), indicating that PDFR signaling is required for PDF neuron control over the network. We conclude that the clock neuron network can produce coherent activity rhythms only when the mismatch between the PDF-positive and -negative neurons is less than ~2.5 hours.

The presence of near 24-hour periodicities despite altered PDF neuron speed suggested that, contrary to the prevailing model, PDF-negative clock neurons have independent control of activity

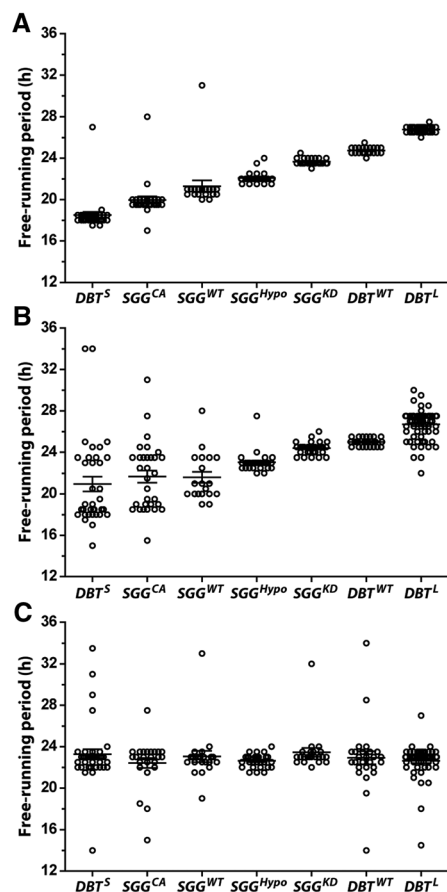


Fig. 1. The PDF-positive clock neurons coherently set free-running periods via PDF signaling over a limited temporal range. (A to C) Scatter plots of the predominant free-running periods of rhythmic flies overexpressing different forms of *DBT* or *SGG* in both PDF-positive and -negative clock neurons (driven by *Clk-GAL4*) (A), or in only the PDF-positive neurons (driven by *Pdf-GAL4*) of WT flies (B) or *Pdf^r* mutants (C). Circles indicate the highest-amplitude free-running period for individual rhythmic flies; lines represent mean $\pm$ SEM (error bars). *DBT^S*, *DBT^{Short}*; *SGG^{CA}*, constitutively active *SGG*; *SGG^{WT}*, WT *SGG*; *SGG^{Hypo}*, hypomorphic *SGG*; *SGG^{KD}*, kinase-dead *SGG*; *DBT^{WT}*, WT *DBT*; *DBT^L*, *DBT^{Long}*. Kruskal-Wallis one-way analysis of variance (ANOVA) reveals a significant difference among groups in (A) and (B) ($P < 0.0001$ for both), but no significant difference among groups in (C) ($P = 0.4829$). h, hours.

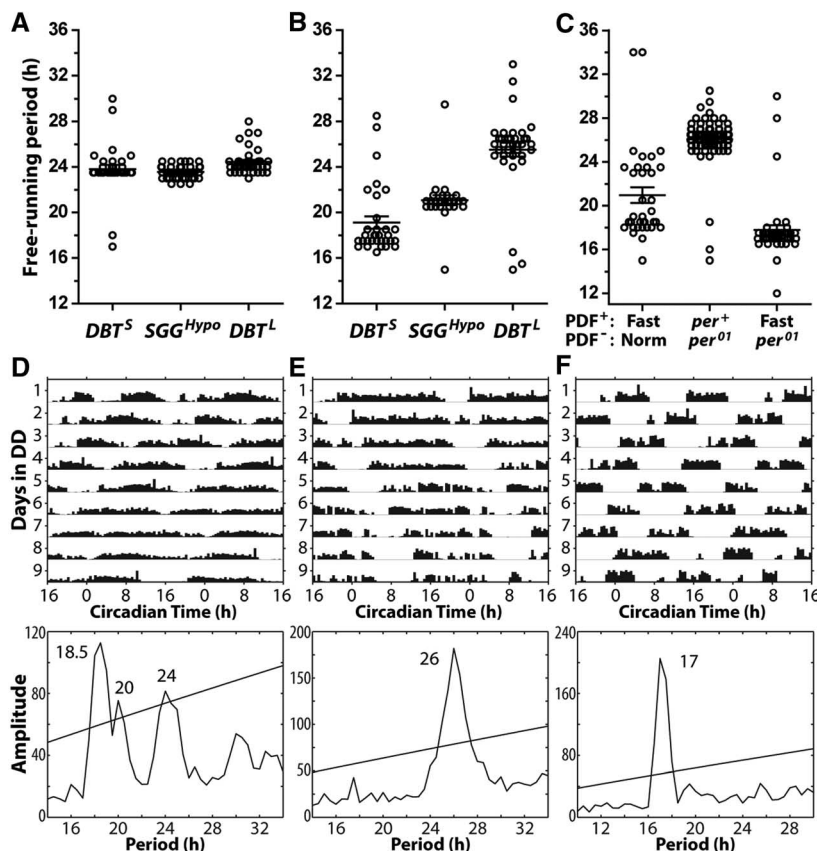


Fig. 2. The PDF-negative clock neurons exert independent control over free-running activity rhythms. (A and B) Scatter plots of the predominant free-running periods of rhythmic flies overexpressing *DBT^S*, *SGG^{Hypo}*, or *DBT^L* only in the PDF-negative neurons (driven by *Clk-GAL4/Pdf-GAL80*) of WT flies (A) or *Pdf^r* mutants (B). Kruskal-Wallis one-way ANOVA reveals a significant difference among groups in both (A) and (B) ($P < 0.0001$ for both). (C) Scatter plots of the predominant free-running periods of rhythmic flies with different compositions of PDF-positive and -negative clock neurons. Specific genotypes are: *per⁺*, *Pdf>DBT^S* for "Fast PDF⁺, normal (Norm) PDF⁻"; *per⁰¹*, *Pdf>PER* for "*per⁺* PDF⁺, *per⁰¹* PDF⁻"; and *per⁰¹*, *Pdf>PER+DBT^S* for "*per⁺* PDF⁺, *per⁰¹* PDF⁻". (D to F) Representative actograms (upper panels) and χ -square periodograms (lower panels) of individual flies with different compositions of PDF-positive and -negative neurons under constant darkness. Genotypes are as follows: (D) *per⁺*, *Pdf>DBT^S*; (E) *per⁰¹*, *Pdf>PER*; and (F) *per⁰¹*, *Pdf>PER+DBT^S*.

rhythms under constant darkness and temperature (DD) (6–8, 11, 12). When we altered the clock speed of PDF-negative neurons, flies displayed increased arrhythmicity and desynchronization and reduced rhythm amplitudes (fig. S7), though the effects were less severe than those seen when PDF-positive neurons were manipulated (Fig. 2A, fig. S8, and table S4) (8). In the absence of PDFR signaling, the PDF-negative neurons determined the pace of free-running rhythms (Fig. 2B, fig. S8, and table S4).

We hypothesized that the phenotypes caused by large clock speed discrepancies (Fig. 1B and figs. S4, S5, and S7) were triggered by conflicts between PDF-positive and -negative clock neurons, both of which drive rhythms. PDF neurons alone are sufficient to drive activity rhythms (6). We predicted that in the absence of clocks in PDF-negative neurons, PDF neurons could coherently drive strong behavioral rhythms at any speed. We restored *period* (*per*) expression only in the PDF neurons of *per⁰¹* mutants (6) (Fig. 2, C and E). When such *per*-rescued PDF neurons overexpressed *DBT^S*, flies displayed a strong ~17-hour period and showed improved rhythmicity, coherence, and rhythm amplitude relative to *DBT^S* overexpression in a wild-type (WT) background (Fig. 2, C and F, and table S5).

Such improvements were also apparent for *DBT^L* overexpression in *per*-rescued PDF-positive neurons (fig. S9 and table S5). We conclude that the ~24-hour periodicities displayed by desynchronized *per⁺* individuals with fast- or slow-running PDF neurons (Fig. 2D and fig. S4) were driven by PDF-negative neurons.

Pigment-dispersing factor signaling is required for the PDF neurons to influence the pace of behavioral rhythms (Fig. 1C and fig. S6), presumably through the resetting of molecular clocks within PDF-negative neurons (8), but only about half of the PDF-negative clock neurons are predicted to express PDFRs (13, 14). Thus, the limited control of the PDF neurons over activity rhythms might be due to a lack of PDF receptivity among PDF-negative neurons. We examined PDF receptivity within the PDF-negative lateral neurons, a fifth small ventral lateral neuron (fifth s-LN_v), and six dorsal lateral neurons (LN_ds) per hemisphere (fig. S1). The fifth s-LN_v responds to bath-applied PDF with increasing cyclic adenosine monophosphate (cAMP) concentration (15). Using the cAMP sensor *Epac1-camps* (15, 16), we found that approximately half of the LN_ds do not display cAMP increases in response to PDF, observing both responding and nonresponding LN_ds within the same brains (Fig. 3, A to D and

G). Restricting the expression of cAMP sensors to the PDFR⁺ LN_ds (14, 17) or the PDFR[−] LN_ds (7), we found that all PDFR⁺ LN_ds (18 neurons from seven brains) displayed cAMP responses to PDF (Fig. 3, E and G), whereas none of the PDFR[−] LN_ds (11 neurons from six brains) responded (Fig. 3, F and G). All LN_ds responded to forskolin, an activator of adenylyl cyclases (Fig. 3H) (18). Thus, PDF modulates only subsets of PDF-negative neurons.

Given such differential receptivity to PDF, we hypothesized that PDF-positive neurons reset the molecular clocks only in subsets of PDF-negative lateral neurons. We visualized PERIOD (PER) protein rhythms in the lateral neuron network of control flies and flies with a large clock speed discrepancy (in this case, flies with the PDF neurons slowed down through expression of *DBT^L*). We chose this manipulation because the internal desynchronization in these flies was usually not accompanied by arrhythmicity (table S2). In control flies, the PDF-positive and -negative lateral neurons all displayed similar phases of PER accumulation on day 4 of constant darkness (DD4) (Fig. 4, A and D). In contrast, there were differences in PER expression between PDF-positive and most PDF-negative lateral neurons in flies with slow PDF neurons (Fig. 4, B and E). Only

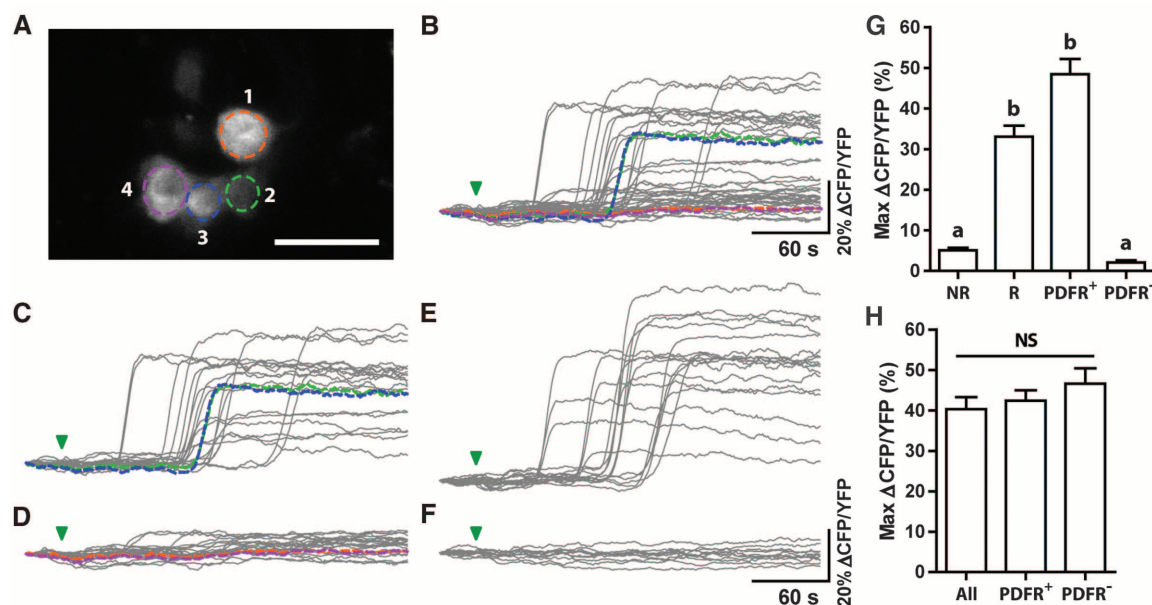


Fig. 3. Pigment-dispersing factor modulates only half of the PDF-negative dorsal lateral neurons. (A) A representative micrograph showing the dorsal lateral neurons (LN_ds) from a *Clk>Epac1-camps* fly brain. Four of the six LN_ds (labeled 1 to 4) were present in the optical section. Scale bar, 5 μm. (B to F) cAMP dynamics of LN_ds in response to bath-applied 10⁻⁵ M PDF peptide (green triangles). Responses of 45 LN_ds imaged from 13 *Clk>Epac1-camps* brains shown in (B) fell into two classes: responsive LN_ds [22 out of 45 (22/45)] that displayed large cAMP increases (>10% change in CFP/YFP ratio) (C) and nonresponsive LN_ds (23/45) (<10% changes) (D). The colored traces in (B) to (D) are from the LN_ds shown in (A) circled with the same color as their plots. All PDFR⁺ LN_ds (18 neurons imaged from seven *Mai179>Epac1-camps* brains) displayed cAMP increases in response to PDF application (E). None of the PDFR[−] LN_ds (11 neurons imaged from six *Clk>cry-GAL80>Epac1-camps* brains)

displayed cAMP increases (F). CFP, cyan fluorescent protein; YFP, yellow fluorescent protein. (G) Summary of maximum cAMP responses of LN_ds to 10⁻⁵ M PDF. NR, nonresponsive LN_ds from (D); R, responsive LN_ds from (C); PDFR⁺ and PDFR[−] are from (E) and (F), respectively. The letters “a” and “b” denote significantly different groups ($P < 0.0001$) by Kruskal-Wallis one-way ANOVA and Dunn’s multiple comparisons test. (H) cAMP responses of LN_ds to bath-applied 10⁻⁵ M forskolin, a direct activator of adenylyl cyclases. “All” represents forskolin responses of LN_ds recorded from *Clk>Epac1-camps* brains, in which the cAMP sensor was expressed in both PDFR⁺ and PDFR[−] LN_ds. The numbers of neurons and brains examined were: All (16 neurons, five brains), PDFR⁺ (12 neurons, five brains), and PDFR[−] (10 neurons, six brains). NS, not significant by Kruskal-Wallis one-way ANOVA and Dunn’s multiple comparisons test. For all histograms, data are presented as mean ± SEM (error bars).

two LN_{dS} per hemisphere were synchronized with the PDF neurons (Fig. 4B). These were the two $PDFR^+$ LN_{dS} that express short neuropeptide F (sNPF) (Fig. 4, G to J, and fig. S10) (19). Synchronization of these two neurons to PDF neurons required PDFR signaling (Fig. 4, C and F). Thus, most PDF-negative lateral neurons were not reset by the slow PDF neurons (Fig. 4, B and E). These uncoupled neurons were probably responsible for the WT periodicities displayed by these flies (Fig. 1B and figs. S4, S5, and S11). Two of the $PDFR^+$ lateral neurons, a single LN_d and the fifth $s-LN_v$ [both of which express ion transport peptide (ITP) (fig. S11) (19)], were

synchronized, not with the PDF neurons, but rather with the $PDFR^-$ LN_{dS} (Fig. 4, B and E), despite their receptivity to PDF. Thus, the slowed PDF neurons reset only two of the seven PDF-negative lateral neurons per hemisphere, despite the fact that four out of seven of these neurons are receptive to PDF, revealing that physiological connections between PDF-positive and -negative neurons do not insure the coupling of their molecular oscillations.

Our results reveal that the PDF-negative lateral neurons consist of at least three functionally and neurochemically distinct oscillatory units: two pairs of $sNPF^+$ / $PDFR^+$ neurons that are strong-

ly coupled to PDF neurons, two pairs of ITP^+ / $PDFR^+$ neurons that are less strongly coupled to PDF neurons, and three pairs of $PDFR^-$ neurons that are not directly coupled to PDF neurons (fig. S11). Each of these oscillatory units is unified by its neuropeptide output and characterized by a distinct mode of coupling to the other oscillatory units (fig. S11). We conclude that the clock neuron network consists of multiple independent oscillators, each capable of orchestrating bouts of activity (fig. S11) and that behavioral rhythms emerge from the interactions of many independent oscillators rather than from a single group of master pacemakers.

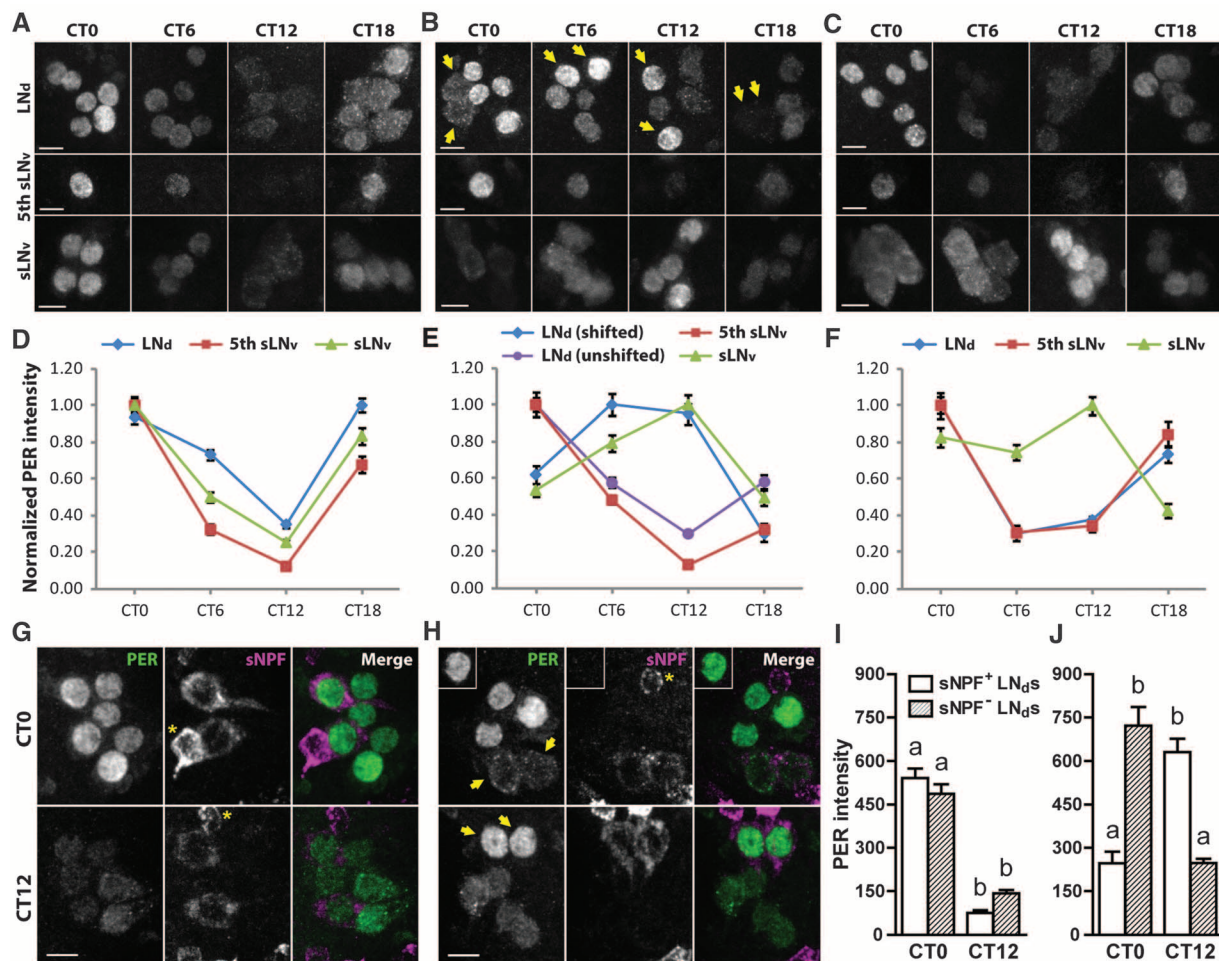


Fig. 4. Physiological connectivity does not ensure molecular clock coupling in the lateral neuron network. (A to C) Immunostaining of PER protein in PDF-positive and -negative lateral neurons across different time points on day 4 of constant darkness (DD4). In *UAS-DBT*⁺ control fly brains, PER accumulated in the PDF-positive ($s-LN_v$ s) and -negative (LN_d s and fifth $s-LN_v$) neurons with the same phase (A). In *PDF>DBT*⁺ flies in which the PDF neurons were slowed down, only two LN_{dS} (marked by yellow arrows) were coupled with the $s-LN_v$ s, displaying a shifted phase of PER cycling relative to the other LN_{dS} (B). In *PDFr*⁺, *PDF>DBT*⁺ flies, all PDF-negative neurons (LN_{dS} and fifth $s-LN_v$) had similar phases of PER cycling, and none were coupled to the uniformly delayed $s-LN_v$ s (C). Scale bars, 5 μ m. (D to F) Quantification of PER immunostaining intensity within lateral neurons of *UAS-DBT*⁺ flies (D), *PDF>DBT*⁺ flies (E), and *PDFr*⁺, *PDF>DBT*⁺ flies (F). The LN_{dS} in (E) were divided into two groups based on their phase differences and quantified separately:

The two LN_{dS} coupled to the $s-LN_v$ s were quantified as “ LN_d (shifted)” and the others as “ LN_d (unshifted).” (G to J) The two shifted LN_{dS} express neuropeptide sNPF. LN_{dS} were coimmunostained for PER and sNPF at CT0 and CT12 on DD4 (CT0 and CT12 correspond to the light-on and light-off times had the 12 hour:12 hour light:dark cycles continued). In *UAS-DBT*⁺ control flies, the two $sNPF^+$ LN_{dS} and four $sNPF^-$ LN_{dS} had similar subcellular PER distribution (G) and expression levels (I) at each of these time points. In *PDF>DBT*⁺ flies, the $sNPF^+$ LN_{dS} differ in their PER distribution [(H), yellow arrows] and intensity (J) from the $sNPF^-$ LN_{dS} . Scale bars, 5 μ m. Asterisks in (G) and (H) indicate $sNPF^+$ cells that are not clock neurons (lack of PER expression). The letters “a” and “b” in (I) and (J) denote significantly different groups ($P < 0.0001$ for both) by Kruskal-Wallis one-way ANOVA and Dunn’s multiple comparisons test. Sample sizes are reported in table S6 for (D) to (F) and in table S7 for (I) and (J). Data are presented as mean $\pm$ SEM (error bars).

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Supplementary Materials

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Materials and Methods

Figs. S1 to S11

Tables S1 to S7

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Quantifying Global International Migration Flows

Guy J. Abel* and Nikola Sander*†

Widely available data on the number of people living outside of their country of birth do not adequately capture contemporary intensities and patterns of global migration flows. We present data on bilateral flows between 196 countries from 1990 through 2010 that provide a comprehensive view of international migration flows. Our data suggest a stable intensity of global 5-year migration flows at ~0.6% of world population since 1995. In addition, the results aid the interpretation of trends and patterns of migration flows to and from individual countries by placing them in a regional or global context. We estimate the largest movements to occur between South and West Asia, from Latin to North America, and within Africa.

Existing data on global bilateral migration flows are incomplete and incomparable because of national statistical agencies not measuring migration or variation in the way migration flows are defined (1–3). Stock data, measured at a given point in time as the number of people living in a country other than the one in which they were born, are more widely available and far easier to measure across countries than are flow data capturing movements over a period of time. This is especially true in regions where the collection of demographic data are less reliable. However, flow data are essential for understanding contemporary trends in international migration and for determining relationships. The discrepancies between the demand for flow data and the availability of migrant stock data have hindered theoretical development and have led to conjectures concerning increases in the overall volume of global migration (4, 5) and shifts in spatial patterns (6).

The demand for bilateral migration flow data that can be the basis for robust comparisons has led researchers to develop indirect estimates. These have been limited to European data, in which flow statistics are plentiful, and have required model-based methods to harmonize reported flows and

impute missing data (7–9). Outside of Europe, global bilateral migrant stock data that capture the size of foreign-born populations in each country—

thus potentially allowing indirect estimations of flows—have only recently become available (10, 11).

Here, we present a set of global bilateral migration flows estimated from sequential stock tables published by the United Nations (U.N.) for 1990, 2000, and 2010 (11). The data are primarily based on place-of-birth responses to census questions, details collected from population registers, and refugee statistics. First, we generated mid-decadal stock tables for the years 1995 and 2005 using a procedure similar to that used by the U.N. to align census and survey data to the beginning year of each decade (11). To quantify the global flow of people over 5-year periods, we then obtained maximum likelihood estimates for the number of movements required to meet the changes over time in migrant stock data, using an iterative proportional fitting algorithm (12). A detailed

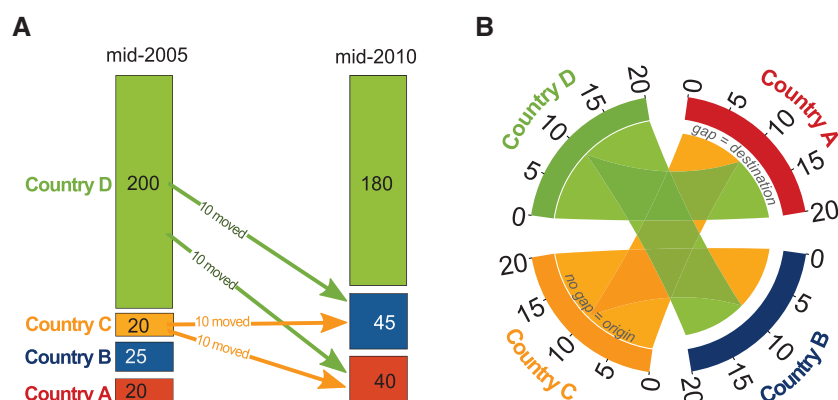


Fig. 1. Linking migrant flow to stock data and visualizing flows via circular plots. (A) The simplified example illustrates our method for estimating 5-year migration flows from changes in stock data between mid-2005 and mid-2010 (details are available in the supplementary materials). The number of people born in Country D and living in Country D (green field) decreased from 200 in 2005 to 180 in 2010. The number of people born in D and living in Country A (red field) increased from 20 to 40, and the number of people living in Country B (blue field) also increased from 25 to 45, but the number living in Country C (yellow field) decreased from 20 to 0. To match these differences in migrant stock data, our model provides an estimate of 20 people moving out of Country C, of whom 10 moved to A and 10 to B, and another 20 people moving out of Country D, with 10 moving to A and 10 to B. (B) The circular plot visualizes the migrant flows estimated in the hypothetical example. The origins and destinations of migrants (Countries A to D) are each assigned a color and represented by the circle's segments. The direction of the flow is encoded by both the origin country's color and a gap between the flow and the destination country's segment. The volume of movement is indicated by the width of the flow. Because the flow width is nonlinearly adapted to the curvature, it corresponds to the flow size only at the beginning and end points. Tick marks on the circle segments show the number of migrants (inflows and outflows).

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discussion of the input data and estimation methodology can be found in the supplementary materials and (13). Our methodology to obtain bilateral flows with a simplified example of changes in stock tables for people born in a hypothetical country is illustrated in Fig. 1A. We produced a comparable set of global migration flows by simultaneously replicating the birthplace-specific estimation procedure for all 196 countries and accounting for changes in populations from births and deaths. Refugee movements are included in our estimates when they are taken into account in the U.N. stock data.

Our bilateral flow estimates capture the number of people who change their country of residence over 5-year intervals, similar to transitions measured over fixed intervals that are recorded by population censuses (14). The net migration totals calculated from our bilateral flow tables match the 5-year net migration data in the U.N. World Population Prospects. A robust comparison with existing bilateral flow estimates for Europe (7–9) is prejudiced by migration being measured as the annual number of movements rather than only a transition over a 5-year period. As the ratio of movements to transitions differs across countries, depending on the amount of multiple and

return moves, there is no simple algebraic solution to convert from one definition to the other (15).

Migrant stock data compare country of birth with country of residence so as to give an estimate of lifetime migration. Compared with our 5-year flow measurement, the longer observation interval provides less detail on the timing of the move (15, 16). Using stock data as a proxy measure for contemporary flows is potentially misleading in the sense that the relative size of immigrant populations does not necessarily correspond to that of migrant flows.

The visualization of global migration flows allows for the visual quantification of directional gross migration flows and the identification of their spatial patterns. Using Circos, a software package widely used in genetics (17), we created circular migration plots (Fig. 1B) to illustrate the complex and dynamic nature of migration. The circular migration plots in Fig. 2 give a snapshot of our flow estimates in 1990 to 1995 and 2005 to 2010 (top) as compared with the U.N. sequential migrant stocks in 1990 and 2010 (bottom), which our estimates are based on (11). Designations of “more developed,” “less developed,” and “least developed” were according to the U.N. Population

Division (11). The patterns of flows during the 1990 to 1995 period are noticeably different from those of the migrant stock data of 1990. Differences between flows and stocks at this aggregated level were not tested with *t* test because such significance tests neglect the array of assumptions behind the estimation model and complexities in the underlying data, and a more fully fledged model-building exercise is beyond the scope of the paper. Fig. 2A depicts a 13% lower share of migration within the developed world and a 6% lower share from the least to less developed world, whereas the share of migration between the least developed countries is 7% higher in comparison with that in Fig. 2C. These differences might reflect sudden changes in the global migration regime driven by the fall of the Iron Curtain and armed conflicts in Asia and Africa. The stock data do not capture these fluctuations in contemporary patterns of movement. The patterns shown in Fig. 2, B and D, are much more similar because migration flows appear to have followed long-term trends captured by stock data.

Contrary to common belief (4–6), our data (Fig. 3) do not indicate a continuous increase in migration flows over the past two decades, neither in absolute or relative terms. According to our estimates, the volume of global migration flows declined from 41.4 million (0.75% of world population) during 1990 to 1995, to 34.2 million (0.57% of world population) during 1995 to 2000. A substantial part of the fall might be accounted for by ceasing of cross-border movements triggered by the violent conflicts in Rwanda and the ending of the Soviet-installed Najibullah regime in Afghanistan. The number of global movements increased by 5.7 million between 1995–2000 and 2000–2005, and by 1.6 million between 2000–2005 and 2005–2010, whereas the percentage of the world population moving over 5-year periods has been relatively stable since 1995.

The size of migration flows within and between 15 world regions in 2005 to 2010 (estimates are in database S1) is shown in Fig. 4. Several migration patterns shown in Fig. 4 are broadly in line with previous assessments based on global stock data (11) and flow data for selected countries published by the U.N. (3, 4, 18, 19). Earlier observations

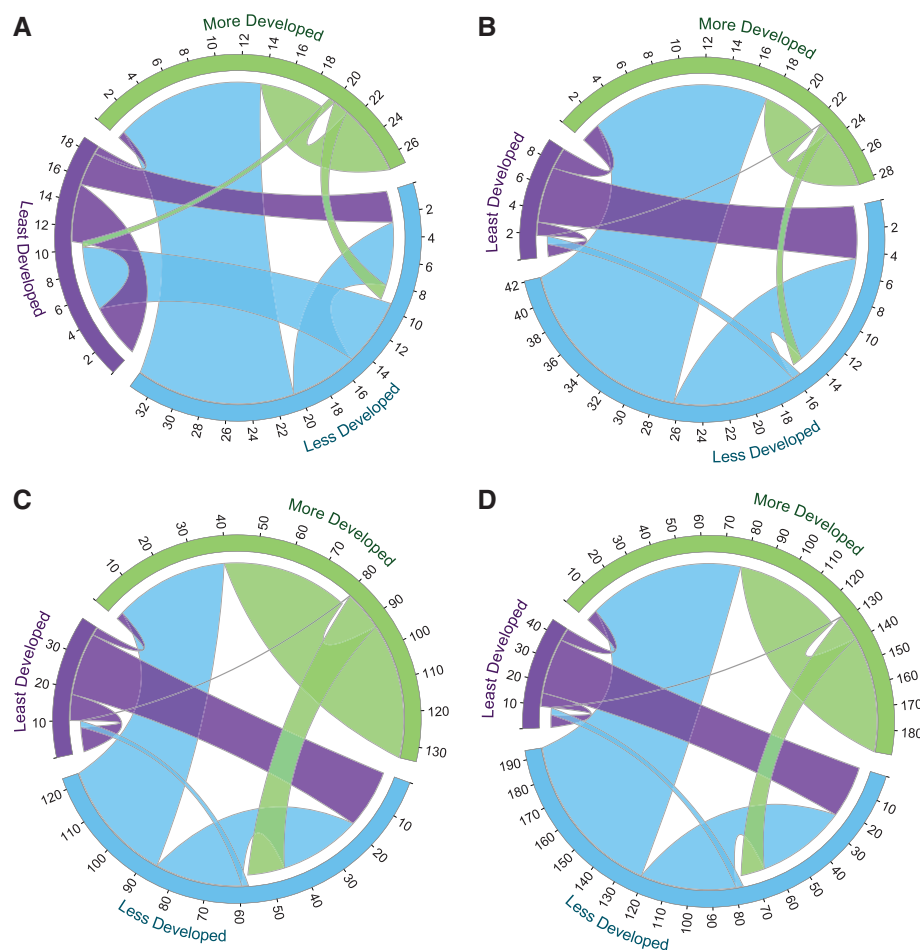


Fig. 2. Comparing estimated migrant flows to stocks in early 1990s and late 2000s. Migration flows between more developed (green), less developed (blue), and least developed (purple) countries. (A) Flows during 1990 to 1995. (B) Flows during 2005 to 2010. (C) Stock data from 1990. (D) Stock data from 2010. Tick marks on the circle segments show the number of migrants (inflows and outflows) in millions.

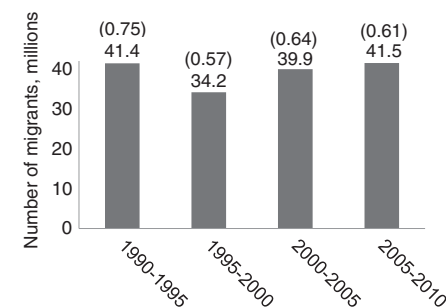


Fig. 3. The global number of international movements between 196 countries in four quinquennial periods, 1990 to 2010. Percentages (shown in parentheses) are calculated by using the world population at the beginning of the period.

include the attractiveness of North America as a migrant destination, the substantial movements from South Asia to the Gulf states in Western Asia, the diverse movements within and between the European regions, and the general tendency for more developed regions to record net migration gains, whereas the less developed countries in Asia, Africa, and Latin America sent more migrants than they received from 2005 to 2010.

A global comparison of migration flows based on our estimates extends these earlier observations and uncovers three striking features of the global migration system. First, African migrants from sub-Saharan Africa (who represent the vast majority of African migrants) appear to have moved predominantly within the African continent. From 2005 to 2010, an estimated 665,000 migrants moved within Eastern Africa, and 1 million people moved within Western Africa. Our data indicate that it is the movements between the member countries of the West African Economic and Monetary Union—especially Ivory Coast, Burkina Faso, and Guinea-Bissau—that drive this pattern (database S2). In contrast, the biggest flow from Western Africa to another continent comprised 277,000 people moving to Western Europe.

Second, migration flows originating in Asia and Latin America tended to be much more spatially

focused than were flows out of Europe. Emigrants from South Asia and South-East Asia tend to migrate to Western Asia, North America, and to a lesser degree, Europe. Migrants from Latin America move almost exclusively to North America and Southern Europe. In contrast, migration to and from Europe is characterized by a much more diverse set of flows to and from almost all other regions in the world.

Third, although the largest flows occurred within or to neighboring regions, the plot depicts numerous flows that go through the center of the circle. These long-distance flows are effective in redistributing population to countries with higher income levels, whereas the return flows are negligible.

Will strong population growth in sub-Saharan Africa lead to mass migration from lower-income countries in Africa to higher-income countries in Europe and North America over the coming decades? Our findings provide evidence for a stable intensity of global migration flows and a concentration of African migration within the continent, with only a small percentage moving to the more developed countries in 1990 to 2010. Therefore, it seems unlikely that if these observed trends persist, emigration from Africa will play a key role in shaping global migration patterns in the future. Nevertheless, human capital and demographic trends create a considerable potential for change

in the global migration system. If, for example, future population growth in sub-Saharan Africa were to be paralleled by a commensurate expansion in education, the growth of a more skilled workforce may lead to an increase in skilled migration from Africa to the more developed world.

In quantifying global migration flows, our data provide a better basis for analyses of the spatial structure of international migration flows that extend beyond the discipline's theoretical and methodological boundaries. A better understanding of the causes and consequences behind current migration patterns may allow for a more informed speculation on future trends.

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Supplementary Materials

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Materials and Methods
Tables S1 to S5
References (20–26)
Databases S1 and S2

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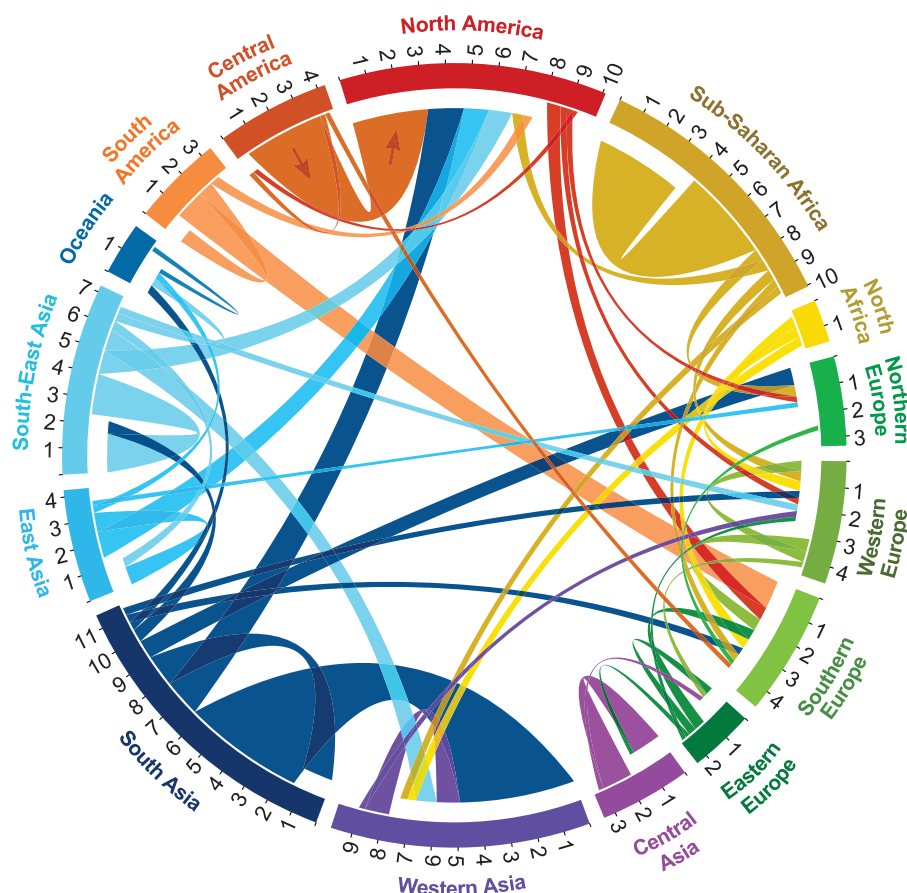
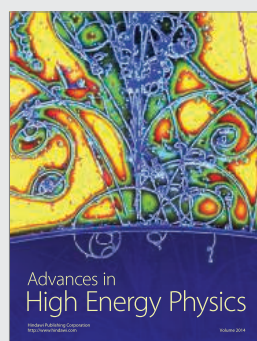
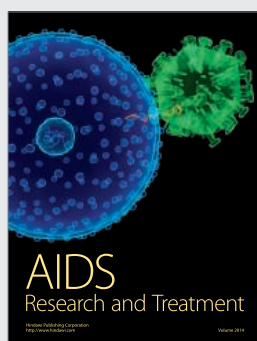
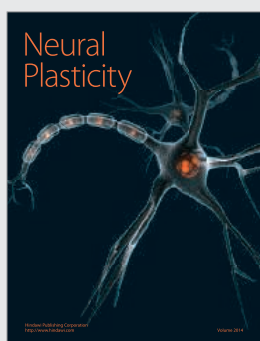
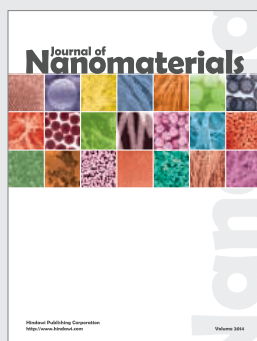
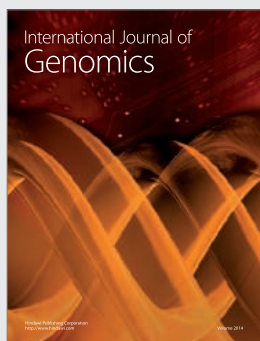
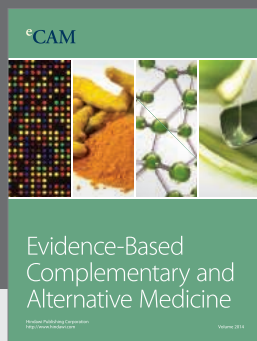
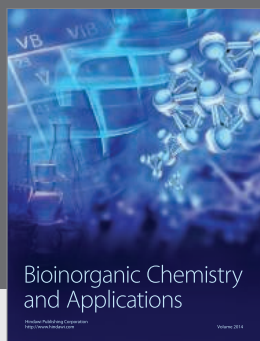
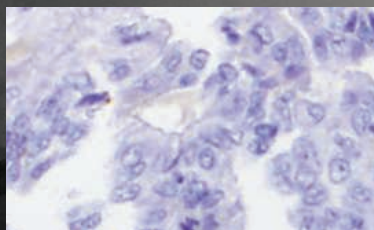


Fig. 4. Circular plot of migration flows between and within world regions during 2005 to 2010. Tick marks show the number of migrants (inflows and outflows) in millions. Only flows containing at least 170,000 migrants are shown.

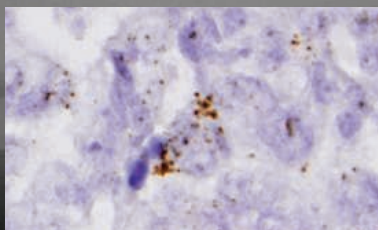


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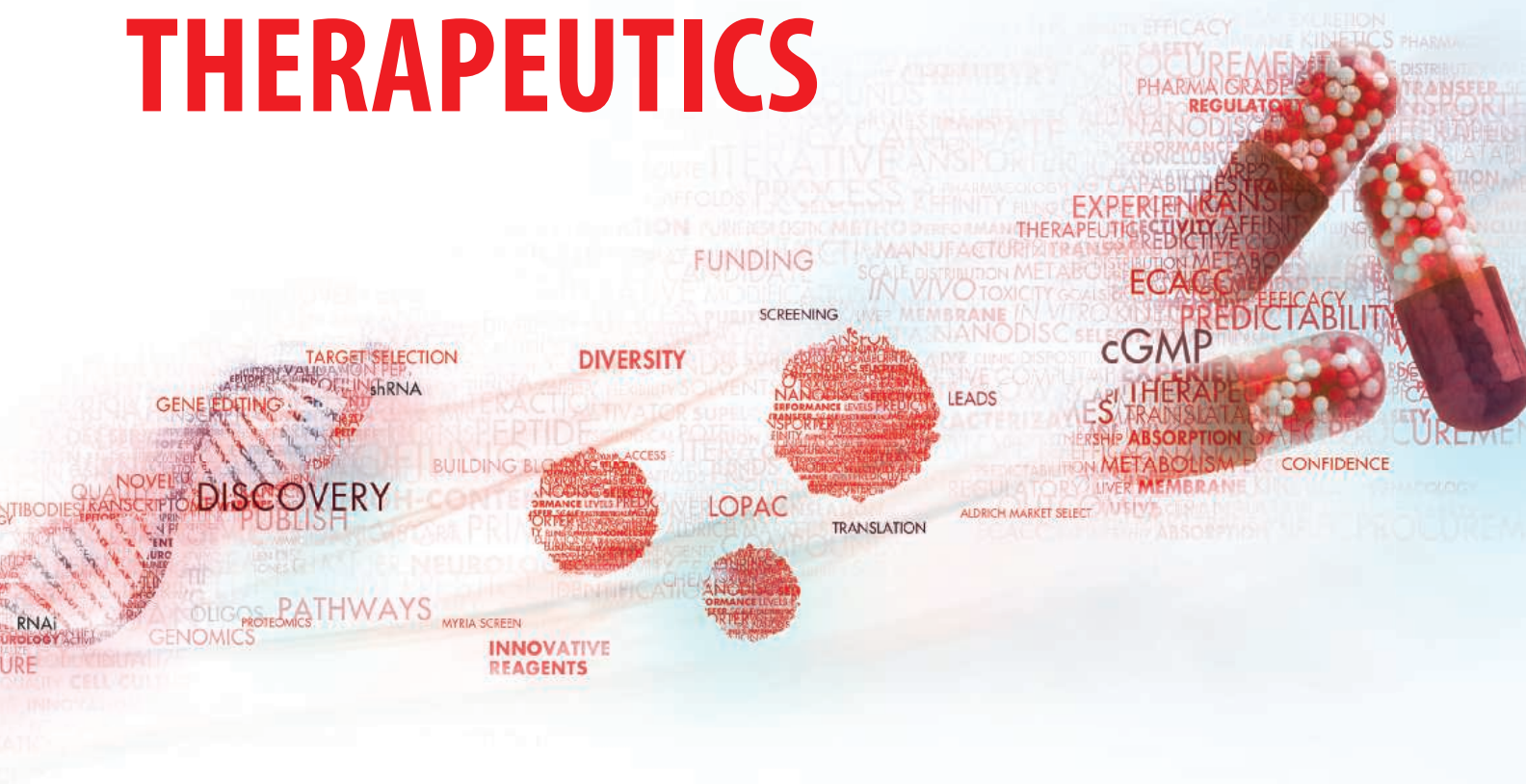


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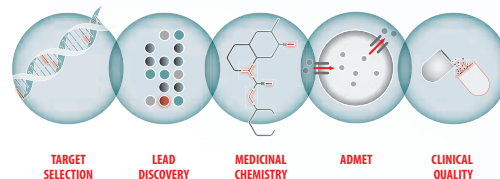
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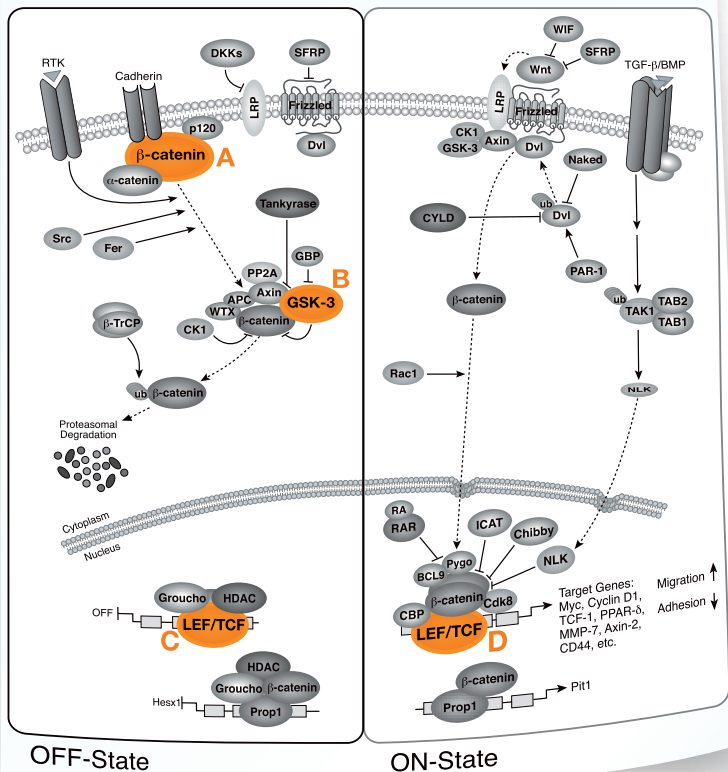
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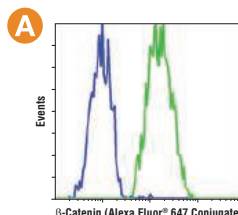
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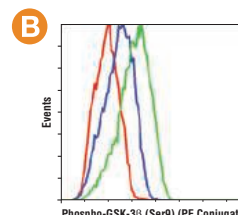


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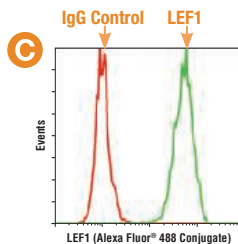
#8466 detects Ser9 phosphorylated GSK-3 β in stimulated cells.



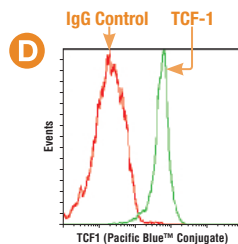
β -Catenin (L54E2) Mouse mAb (Alexa Fluor[®] 647 Conjugate) #4627: Analysis of NCI-H28 cells (blue) and HeLa cells (green).



Phospho-GSK-3 β (Ser9) (D85E12) XP[®] Rabbit mAb (PE Conjugate) #8466: Analysis of NIH/3T3 cells treated with hPDGF-BB #8912 and λ phosphatase (red), untreated (blue), or treated with hPDGF-BB #8912 only (green).



LEF1 (C12A5) Rabbit mAb (Alexa Fluor[®] 488 Conjugate) #8490: Analysis of Jurkat cells using LEF1 (C12A5) Rabbit mAb (Alexa Fluor[®] 488 Conjugate) (green) compared to Rabbit (DA1E) mAb IgG XP[®] Isotype Control (Alexa Fluor[®] 488 Conjugate) #2975 (red).



TCF1 (C63D9) Rabbit mAb (Pacific Blue[™] Conjugate) #9066: Analysis of Jurkat cells using TCF1 (C63D9) Rabbit mAb (Pacific Blue[™] Conjugate) (green) compared to Rabbit (DA1E) mAb IgG XP[®] Isotype Control (Pacific Blue[™] Conjugate) #9078 (red).



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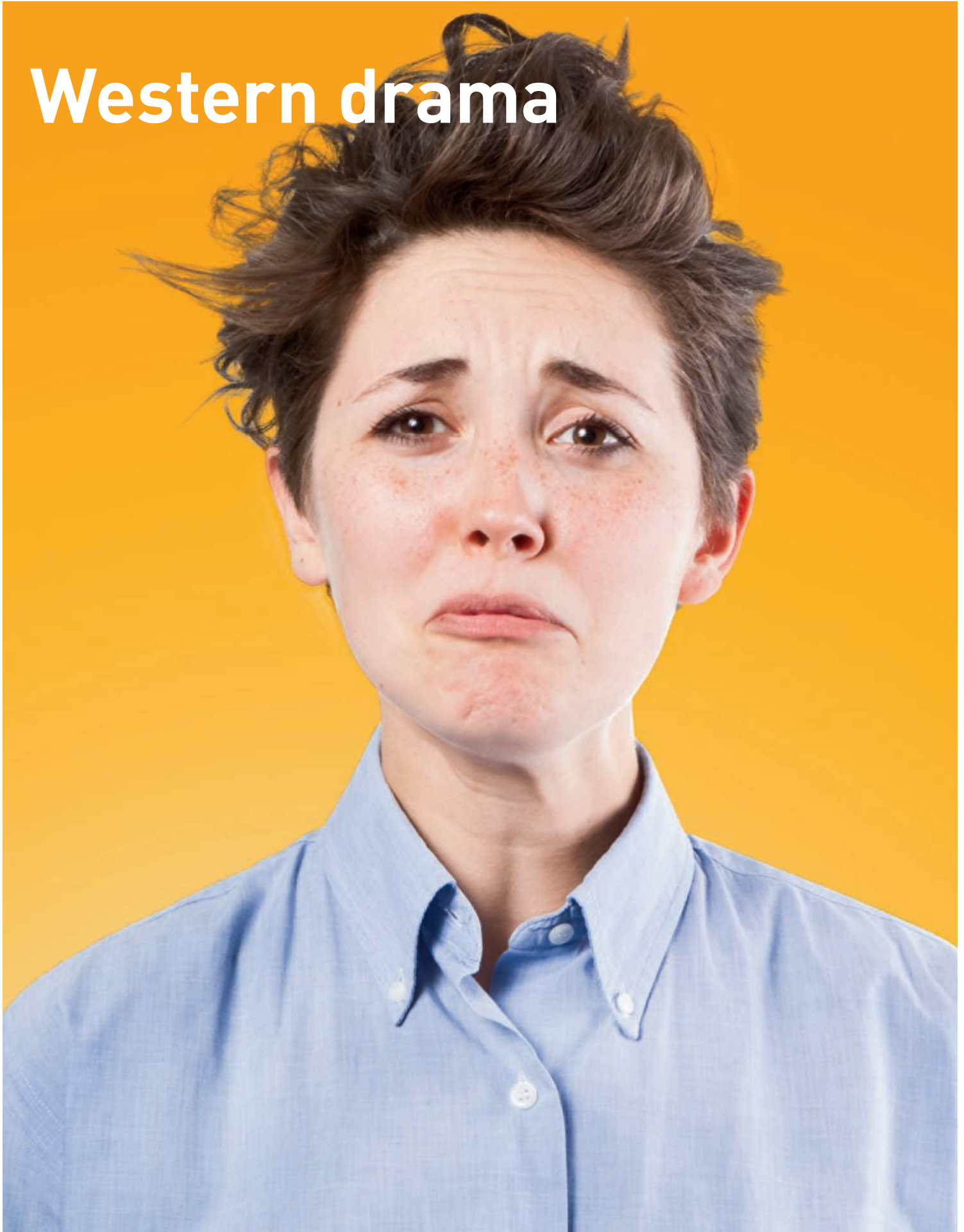
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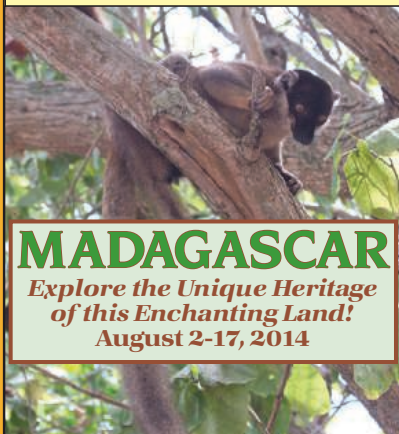
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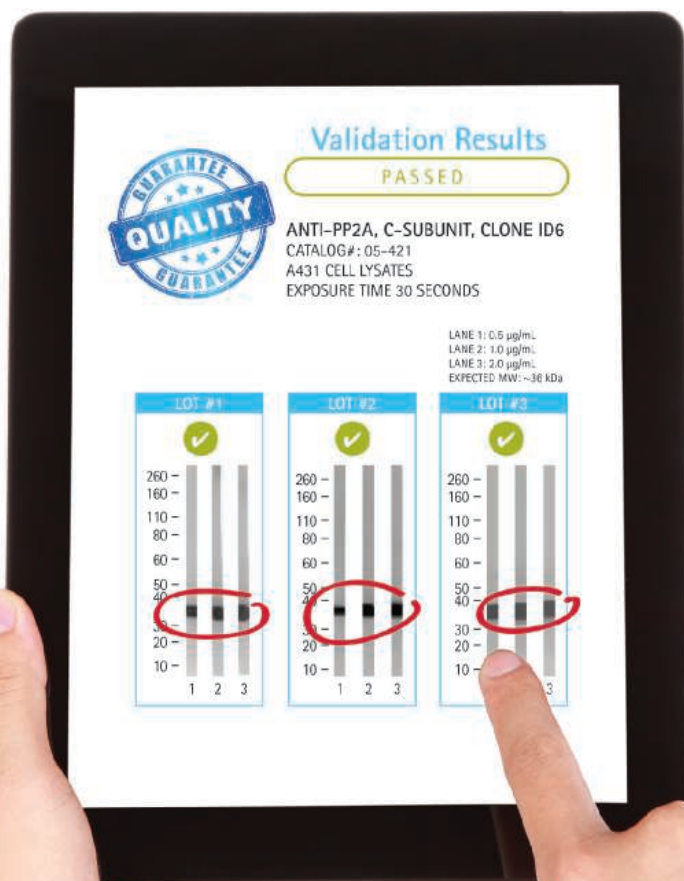
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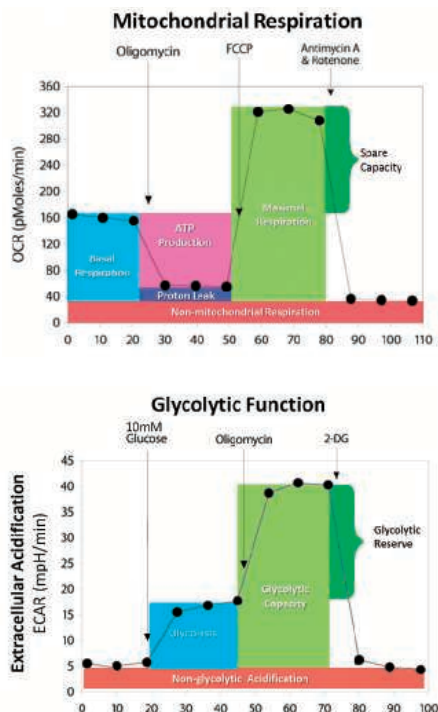
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Mallinckrodt Distinguished Professor of Pathology, Beth Israel Deaconess Medical Center

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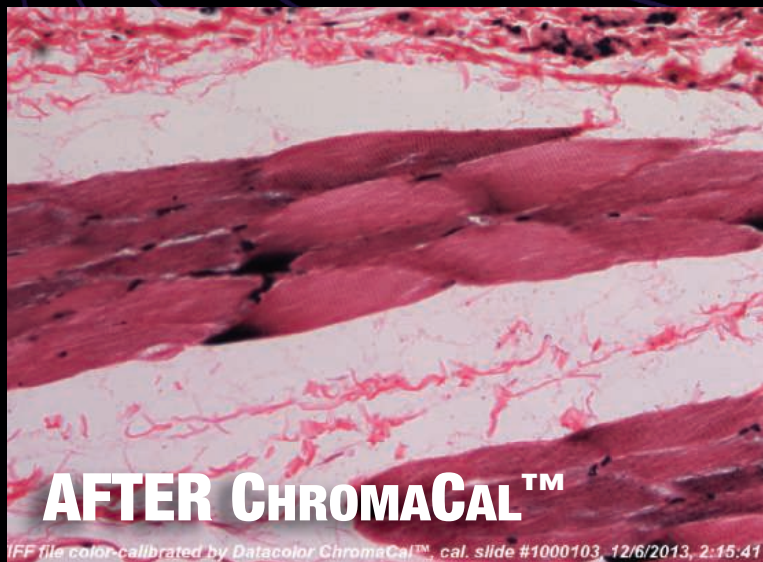
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Initiating a nation-wide effort to boost Japan's scientific landscape, the Ministry of Education, Culture, Sports, Science and Technology (MEXT) has launched the Program for Promoting the Enhancement of Research Universities, which exemplifies new trends in research funding in Japan. Twenty-two academic institutions have been singled out from the hundreds of Japanese universities and institutes to lead Japan's efforts to stay at the forefront of science.

These now-elite institutions were chosen via a nontraditional, top-down selection process—a rarity for these types of initiatives in Japan. The funding process is simple: set strategic research targets and priorities, conduct preliminary surveys for potential researchers and institutes most likely to achieve the goals, and provide generous funding to small numbers of top scientists for long periods of time. Representatives from the smaller and newer institutes assert that this unusual metrics-based assessment has enabled them to take on projects that would be unheard of with the traditional bottom-up, proposal-based funding procedures, which can favor larger universities.

After being awarded the prestigious status and funds, these 22 universities are now responsible for developing Japan's scientific infrastructure and making the necessary infrastructure-related reforms to strengthen their research portfolios. This includes hiring research administration managers, recruiting top researchers from overseas, and analyzing global scientific trends in order to formulate new research strategies. The financial support to accomplish these goals ranges from US\$2 million to US\$4 million annually for 10 years, with a midterm assessment after five years that promises funding cuts in cases of poor performance.

The institutes selected (Table 1) include well-established former Imperial Universities (Hokkaido, Tohoku, Tokyo, Nagoya, Osaka, Kyoto, and Kyushu) as well as three smaller, newer institutes (the University of Electro-Communications, Toyohashi University of Technology, and Nara Institute of Science and Technology) and two large, research-based private universities (Keio and Waseda).

Intriguingly, despite having produced the largest number of Nobel Prize Laureates in Asia, Japan is not well represented in world university ranking tables. For example the 2013–2014 *Times Higher Education World University Rankings* lists

only two Japanese universities in the top 100, on par with Singapore, but behind South Korea and China with three each.

This lack of visibility and representation in world rankings is a source of considerable discussion in academic circles in Japan. Many of the 22 universities have declared that part of the funding from the MEXT program will be used to pull their institutes into the top 10 of the rankings tables by the end of the 10-year program, with others aiming for a more modest goal of being within the top 100.

Some of the major challenges these universities will be faced with overcoming are: the low birth rate that has led to major decreases in the number of high school children qualified to enter university; severe constraints on research funding for universities, and the need for greater internationalization and visibility.

THE DEMOGRAPHIC LANDSCAPE

Japan's coffers are feeling the pinch. As of May 2013 there were approximately 770 universities in Japan: 86 national institutes, 83 prefecture or city-run, and 601 private. They all rely on government subsidies to run education and research programs. Financing university education and research is putting a huge burden on government resources that are also being strained by increases in medical costs due to the rapidly aging population (24.1% are over 65 years old), the costs for reconstructing the Tohoku region following the devastating earthquake and tsunami in 2011, and demands for investment in trillion-yen international “big-science” projects.

Government subsidies are crucial for the majority of universities to exist. The falling birthrate in Japan has led to excess capacity within the education system, causing an increase in bankruptcies of private universities and forcing national universities to introduce early retirement plans to reduce personnel costs.

The MEXT program sends a clear message to university administrators that MEXT cannot continue to subsidize all the universities in Japan. Future funding for research will be limited and based on objective statistics, such as citations, revenue from technology transfer, and international rankings. This funding must be used to improve university infrastructure, hire top researchers and managers, and engender innovation to improve Japan's global competitiveness in science.

UNIVERSITY MANAGEMENT

In 2004, the Japanese government overhauled the management of national universities by introducing a more corporate-like structure and giving presidents (elected by faculty) more autonomy to hire staff, determine salaries, and set long-term goals for education and research. Furthermore, MEXT started reducing government financial support to national universities by 1% per year. This has led to financial dilemmas for universities that are unable to fill the resulting funding gap with other sources of income.

Another common theme is revamping the role of university research administrators (URAs) to undertake a multitude of tasks including supporting researchers in procuring funding, interacting with industries to license university intellectual property, and analyzing trends in research themes to devise strategies for future, unexplored areas of research. Some universities plan to hire as many as 40 URAs as part of the program in order to take some of the administrative pressure off research faculty. More senior URAs will likely be recruited from the private sector, but many universities will tap into the postdoctoral pool, training them as part of new URA career paths. It's notable that Japan has a huge number of postdocs looking for permanent posts, particularly in the life sciences.

Table 1. Universities and research institutes selected for the MEXT Program for Promoting the Enhancement of Research Universities. (Numbers indicate annual funding in millions of Yen.)

Hiroshima University (300)
Hokkaido University (200)
Kobe University (200)
Kumamoto University (200)*
Kyoto University (400)*
Kyushu University (300)
Nagoya University (400)*
Nara Institute of Science and Technology (300)*
Okayama University (200)*
Osaka University (300)
Tohoku University (400)
Tokyo Institute of Technology (300)*
Tokyo Medical and Dental University (300)
Toyohashi University of Technology (200)*
University of Electro-Communications (300)*
University of Tokyo (400)
University of Tsukuba (300)*

Private Universities

Keio University (200)*
Waseda University (300)*

Other Institutes

High Energy Accelerator Research Organization (300)
National Institute of Informatics (300)
National Institutes of Natural Sciences (300)

*universities featured in this advertorial

INTERNATIONALIZATION

The projects in the MEXT program contain some common themes and goals. The universities have all made internationalization or *kokusaika* one of their highest priorities. *Kokusaika* has different meanings to different people. Here the interpretation is to increase the number of overseas researchers and students, and improve international collaboration by implementing various plans: increasing accommodation facilities for overseas researchers, introducing English language courses for students, training administration staff to produce bilingual documents, and introducing new salary scales commensurate with institutes in the United States and Europe.

So what are the challenges in hiring foreign staff? Short-term stays in Japan for young researchers can be highly rewarding and valuable for boosting their later employability, but establishing a long-term career may be more challenging due to potential family-related issues such as securing a job for a spouse and a good education for accompanying children.

The Japanese language can also be a stumbling block. Foreign researchers may feel isolated and conducting independent research can be extremely taxing; simple exercises such as ordering equipment and taking part in departmental meetings are difficult without reasonable proficiency in Japanese. Even with excellent language skills, foreign academics are unlikely to be awarded top positions such as dean and president in Japanese academia.

The emphasis on *kokusaika* reflects concerns about Japanese researchers becoming too inward looking. Several years ago journalists coined the term “Galapagos Syndrome” to describe the situation, exemplified by highly advanced Japanese mobile telephones that were incompatible with systems in other countries and thus globally irrelevant.

Improving global rankings and attracting top-class foreign researchers are two of the main challenges ahead as the MEXT program moves forward. Needless to say, project managers are well aware of them and have no doubt devised solutions to resolve these issues to achieve their goals. Only time will tell if the 22 institutes will each achieve their goal of becoming globally competitive. The main issue will be the degree to which Japan's academic community is recognized for its contribution to the global creation of knowledge. Perhaps this is the true meaning of *kokusaika*.

Adarsh Sandhu is a freelance science writer based in Tokyo, Japan.





University of Tsukuba

A Hub of Academic and Industrial Collaboration



Michiyoshi Ae



Skiing Practice as Outdoor Education



Judo Olympians Training

The University of Tsukuba campus is located at the heart of Tsukuba Science City, about 45 minutes north of Tokyo on the Tsukuba Express train. The modern structure of the university was established in 1973 after the reorganization of its predecessor, the Tokyo University of Education, whose roots go back to 1872. The huge campus—similar in size to New York's Central Park—is home to approximately 16,500 students and 4,100 faculty and administrative staff. The university offers a comprehensive curriculum including arts and social sciences, physical education and sports sciences, physical sciences and engineering, and medicine. Distinguished scholars affiliated with the university include Nobel Laureates Leo Esaki (Physics, 1973), Hideki Shirakawa (Chemistry, 2000), and Sin-Itiro Tomonaga (Physics, 1965).

Looking more broadly, Tsukuba is one of the world's largest science and knowledge-based regions in the world. It has 32 research and academic institutions, approximately 20,000 researchers, and more than 7,000 foreign workers.

In recognition of the technological importance of the Tsukuba region, the Japanese Central Government and local authorities designated Tsukuba City and its peripheral regions as an "International Strategic Zone" in 2011. Managed by the Tsukuba Global Innovation Promotion Agency, the new zone was set up to act as a hub for academia-industry collaboration to foster innovative solutions to major problems facing Japan, such as the declining birth rate, an aging population, and the need for long-term energy resources.

Yasuo Miake is vice president and executive director of re-

search affairs at the University of Tsukuba, and one of the central members of the team managing the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Program for Promoting the Enhancement of Research Universities. "Our strategy for running the MEXT program has three main features: introducing an international tenure-track program, headhunting top-class researchers from overseas, and increasing the number of university research administrators from the 11 we have at present to 30 within the next five years," explains Miake. "Importantly, we want to assure our researchers that they will have more time to conduct high-quality research by reducing their time spent on administration and paperwork." Miake also stresses the central roles of the Center for Computational Sciences, the Life Science Center of Tsukuba Advanced Research Alliance, and the Center for Cybernetics Research in achieving the goals of the MEXT program (see sidebars below).

Michiyoshi Ae, executive director and vice president in charge of education affairs, who is an expert in sport biomechanics, adds that in addition to traditional research in the basic and applied sciences, the university is a powerhouse for sports and physical education, having produced scores of Olympic medalists and professional sportsmen. "We are now working with MEXT to train sports coaches, physical educators, and sport scientists in Africa and Asia." Research at the University of Tsukuba is truly global and multidisciplinary," concludes Ae.

Tsukuba International Strategic Zone:

www.tsukuba-sogotokku.jp/en/

Center for Computational Sciences

"Research at the Center for Computational Sciences [CCS] consists of both hardware development and supercomputer construction, and applies these resources to conduct research in areas such as particle physics, astrophysics, weather forecasting, and condensed matter physics," says **Masayuki Umemura**, director of CCS.

CCS has an impressive record of achievements, including completion of the Computational Physics by Parallel Array Computer

System (CP-PACS) in September 1996. At the time CP-PACS was the fastest supercomputer in the world. A more recent development is the 95.4 teraflops T2K Tsukuba System, a large-scale, general-purpose computer cluster that was developed through a collaboration with the University of Tokyo and Kyoto University as part of the T2K Open Supercomputer Alliance. Construction of the 1.166 petaflops (1,166 teraflops) Highly Accelerated Parallel system for Computational Sciences (HA-PACS) in col-



Masayuki Umemura

laboration with the U.S.-based company Cray is another recent achievement because it uniquely



incorporates graphics processing units used for games in personal computers.

New initiatives based on the computing power at CCS include the High Performance Computer Infrastructure (HPCI) Strategic Program, a multi-institute collaborative project. The HPCI includes use of the 8.162 petaflops K-Computer and aims to reveal “the origins and structure of materials and the cosmos.” Notably, CCS was awarded the Gordon Bell Prize twice for work using the K-computer,

for their joint research on electronic states in semiconductor nanowires in 2011, and for their simulations of the gravitational forces acting simultaneously on one trillion astrophysical (dark matter) particles (2012).

Umemura explains that collaborative research with planetary scientists has yielded new insights into the existence of so-called left-handed amino-acids and, by extension, into the origins of life after the big bang. Other research using the simulations has helped scientists

better understand interstellar turbulence and the formation of stars and planets.

“We currently have a staff of 33 full-time researchers and 14 collaborators,” says Umemura. “The funding from the MEXT program will be used to hire four young researchers as part of the university’s international tenure-track program.”

Center for Computational Sciences:
www.ccs.tsukuba.ac.jp/eng/

Life Science Center, Tsukuba Advanced Research Alliance

The Life Science Center of Tsukuba Advanced Research Alliance (LS-TARA Center) was originally launched as the Tsukuba Advanced Research Alliance (TARA) in May 1994 by the former president of the University of Tsukuba, Leo Esaki. TARA was a research platform used to conduct interdisciplinary research in cutting-edge research areas such as life science and nanomaterials, through collaborations between academia, industry, and government.

In March 2010, TARA was reorganized to create the LS-TARA Center, reflecting the university’s determination to strengthen its contribution to research in the life sciences, including fast and inexpensive genome sequencing, animal cloning, and stem cell research.

Akiyoshi Fukamizu is vice director of the LS-TARA Center and is internationally renowned for his research on a mouse model

used to study high blood pressure during pregnancy. “Our findings on pregnancy-induced hypertension [PIH] in mice were a classic case of serendipity,” explains Fukamizu. “We had spent many years studying genes that encoded proteins involved in the regulation of blood pressure and the role of the renin-angiotensin system.” Breeding genetically altered mice carrying certain combinations of the renin-angiotensin system proteins led to the discovery of pregnant mice exhibiting PIH-related symptoms. The publication of these results in *Science* in 1996 led to worldwide discussion of their implications and the utility of having a mouse model to study PIH, which occurs in 10% of women. “These animals do not exist in nature because they die,” explains Fukamizu. “This is an important model for finding a treatment for PIH.”



Akiyoshi Fukamizu

Future research at LS-TARA will receive extensive support from MEXT, in particular for hiring at least 10 principle investigators with expertise in areas such as genetics, chemical communication, and metabolism. “We are actively recruiting new research staff,” says Fukamizu. “I am busy travelling overseas to interview candidates to fill these new, exciting posts.”

Life Science Center of Tsukuba Advanced Research Alliance:
www.tara.tsukuba.ac.jp/about/aboutENGLISH.html

Center for Cybernics Research

Robot Suit HAL (Hybrid Assistive Limb) is the creation of **Yoshiyuki Sankai**, director of the Center for Cybernics Research, born out of his passionate desire to help people around the world. “HAL is the world’s first cyborg-type robot that treats, supports, and expands the physical capabilities of humans. We are also developing this device to be used for medical treatment,” says Sankai. “This powered exoskeleton is already in use at over 170 medical and welfare institutions in Japan. Clinical trials with HAL are being carried out in Japan as well as at BG-University Hospital “Bergmannsheil” in Germany and the Karolinska Institute in Sweden, and HAL has been granted CE0197 certification in Europe. Furthermore, a neuro-rehabilitation center in Germany is providing treatments using HAL that are now covered by national workers insurance. This is the realization of one of my childhood dreams: to build robots to help humans.”

HAL is manufactured by Cyberdyne Inc., a venture company set up by Sankai and now ISO 13485 certified. The exoskeleton is controlled by a combination of special algorithms,

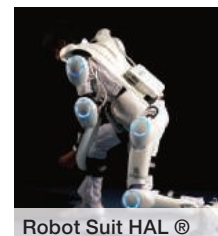
a Cybernic Voluntary Control (CVC) system, and a Cybernic Autonomous Control (CAC) system. The CVC system uses the wearer’s own intentions (i.e., bioelectrical signals from brain to muscle) detected by the sensors, while the CAC system operates based on a preprogrammed system determined by analysis of basic motion patterns and movement mechanisms in humans, in case the bioelectrical signals are weak. A dual systems operation mode enables support of natural physical movements in accordance with the wearer’s intentions.

Sankai explains that the success of HAL is a testament to the “flat nature of the organization” at the University of Tsukuba and its mission to “open new frontiers.” Says Sankai: “One of the unique characteristics of the university is that professors all receive the same annual university funding, irrespective of whether they are full or assistant professors. We are treated equally and given every opportunity to start new, innovative projects, as I did when I started work on robotics in my younger days here.”

Important recent developments include the electronically powered exoskeleton being the



Yoshiyuki Sankai



Robot Suit HAL ®

world’s first ever device of its kind to receive a global safety certificate (ISO/DIS13482). In addition, in August 2013 the potential of HAL was recognized when it was awarded the European Conformity (Conformité Européenne, CE) mark for medical devices.

Sankai is confident that HAL will help resolve many problems related to Japan’s rapidly aging society and health care in general. “This is just the beginning. We are initiating clinical tests and new experiments in areas such as testing new drugs for treating polio. We have full support from the university and the Japanese government as part of this program and other projects.”

University of Tsukuba Center for Cybernics Research:
www.first.ccr.tsukuba.ac.jp/english/



Tokyo Institute of Technology

Aiming to Become One of the World's Top 10 Research Universities

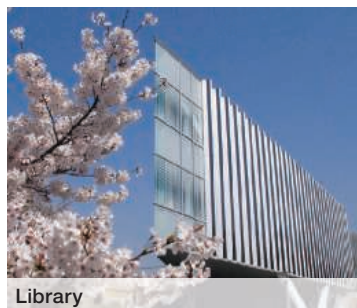
Tokyo Institute of Technology (Tokyo Tech) is Japan's premier science and technology research university. Established in 1881 as the Tokyo Vocational School, the institute currently has approximately 1,200 faculty, 10,000 students, and an annual revenue of 46.7 billion Yen (US\$445 million).

Tokyo Tech's strengths include physics, material science, earth sciences, and computer science. Its internationally acknowledged contributions to science and technology include the early transmission of an image via a cathode ray tube by Kenjiro Takayanagi in 1926; the 1967 invention of electrically conductive plastic by Hideki Shirakawa (awarded the 2000 Nobel Prize in chemistry for this research); and deployment of Tsubame 1.0 in 2006, one of the fastest supercomputers in the world at the time.

Yoshinao Mishima, president of Tokyo Tech talks about how the mission of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Program for Promoting the Enhancement of Research Universities will impact Tokyo Tech: "Our immediate goal," he explains, "is to be in the top 100 in the THE [Times Higher Education] world university rankings. My long-term goal for the university is to be one of the top ten research universities in the world by 2030. I believe that the program's international benchmarking provides a useful measure for the quality of our research and education."



Yoshinao Mishima



Library

The reforms will be led by new university research administration (URA) staff, hired with part of the \$3 million in annual funding from the MEXT program. "The URAs will analyze and benchmark our research to quantify our strengths and weaknesses, and recommend new areas

of research to pursue," says Mishima. Three major projects being undertaken at Tokyo Tech include research at the Earth-Life Science Institute, the Materials Research Center for Element Strategy, and the Environmental Energy Innovation Building (see sidebars below).

The initial goals being addressed are increasing international collaborative research, inviting more overseas scientists to conduct research at Tokyo Tech, and improving the citation levels of papers published by Tokyo Tech researchers. "Structural reforms such as sufficient accommodation for overseas researchers must be complemented with administrative reforms, such as bilingual documentation," stresses Mishima. While positive changes are already under way, Mishima acknowledges that "there are many challenges in the years ahead."

Tokyo Institute of Technology:
www.titech.ac.jp/english/

Earth-Life Science Institute

Recent advances in astronomical instrumentation and technology have led to regular reports on the discovery of extrasolar planets, or exoplanets, that orbit stars outside of our solar system. The goal of such research is to find other habitable planets, and possibly discover other forms of life there.

Complementing the research on exoplanets, **Kei Hirose** and colleagues at the Earth-Life Science Institute (ELSI) at Tokyo Tech are investigating the environmental factors that enabled life to develop on Earth. "ELSI was established



Hirose Talking with ELSI Researcher

in 2012 to answer this very basic question," says Hirose, director of ELSI. "Our findings will shed light on the possibility

of finding life on other planets."

"Phosphorous is essential for life on Earth, but it is a scarce element in the universe," explains Hirose. "How was phosphorous made available to the Earth's earliest life? Were there unique rocks on the early Earth that were enriched in phosphorous? Under what environmental conditions did DNA and RNA—molecules that have phosphate backbones—form and give rise to early life on Earth? These are some of the fundamental questions we will try to answer."

ELSI is one of nine World Premier International (WPI) Research Center Initiatives funded by MEXT. Some of the specific areas of research include investigating how the Earth was formed and the origin of life, how life evolved and why the atmosphere contains oxygen, the effects of "galactic events" on the Earth's environment, and construction of a center of bioplanetology to investigate the possibility of life on other planets.

ELSI is an international research institute where, once fully staffed, more than 30% of the researchers will be from overseas.



Kei Hirose

It includes a global network of collaborators and has satellite centers at the Geodynamics Research Center, Ehime University, Japan; the Institute for Advanced Study, Princeton University; and the Origins of Life Initiative at Harvard University. It also supports partnerships with the Japan Agency for Marine-Earth Science and Technology and the Institute of Space and Astronautical Science (part of JAXA) in Japan.

Earth-Life Science Institute:
www.elsi.jp/en



Materials Research Center for Element Strategy

Hideo Hosono—known for his invention of the high mobility oxide thin film transistor for flat panel displays and iron-based superconductors—is the director of the Materials Research Center for Element Strategy (MCES). The center was established at Tokyo Tech in 2012 as an independent organization to run projects at the Tokodai (Tokyo Tech) Institute for Element Strategy (TIES), also led by Hosono. Construction of a dedicated MCES building on the Suzukakedai Campus of Tokyo Tech is scheduled to finish in March 2014.

Hosono is also the project leader of the Accelerated Innovation Research Initiative, ACCEL—a five year, approximately US\$15 million project funded by the Japan Science and Technology Agency (JST). The project will look into ways of exploiting the chemical and electronic properties of electrides (a compound in which an electron serves as an anion) and develop proof-of-concept strategies for the following three areas:

(1) Development of catalysts for the realization of small, on-site, low energy production plants for ammonia, because of its importance for producing fertilizers and as an energy carrier for hydrogen

(2) Realization of large-area, low-power consumption organic electroluminescent devices with efficient ways of injecting electrons



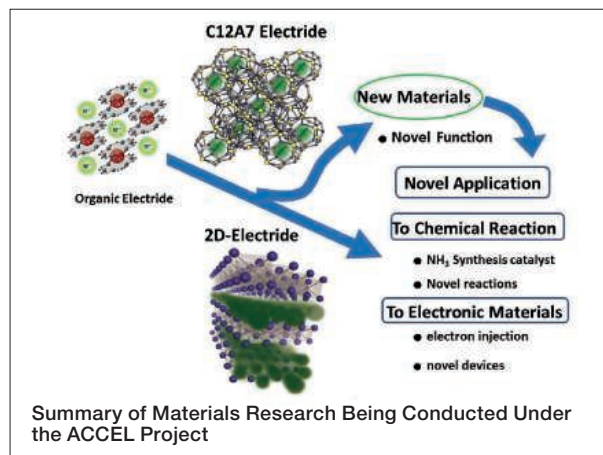
Hideo Hosono

into the active layers

(3) Development of a deeper understanding of the materials science of electrides.

The project builds on the success of previous work, including an Exploratory Research for Advanced Technology (ERATO) project in which a metallic oxide, mayenite [$12\text{CaO} \cdot 7\text{Al}_2\text{O}_3$ (or C12A7)], was converted into a conductor, as well as research performed under the Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST). This research led to some important discoveries, including the synthesis/decomposition of ammonia by ruthenium nanoparticles loaded on the inorganic electride, C12A7:e^- .

“This research is part of a national concept called the Element Strategy Initiative [ESI],” explains Hosono. “This ESI project is being



carried out by 20 researchers located at Tokyo Tech, the National Institute of Materials Science, the High Energy Accelerator Research Organization, and the University of Tokyo,” notes Hosono. “A major objective of our ACCEL project is to seek alternatives for the Haber process, the main method for producing ammonia, which was invented around a hundred years ago. Ammonia is important because it continues to be a vital component for sustaining modern society.”

Materials Research Center for Element Strategy:
www.mces.titech.ac.jp/en/

Environmental Energy Innovation Building

Manabu Ihara applied his expertise in energy conversion chemistry in the construction and management of the Environmental Energy Innovation Building (EEI), Tokyo Tech's flagship energy-conscious research facility, in cooperation with architectural specialists, Yoshiharu Tsukamoto and Toru Takeuchi.

“This building was designed to be nearly self-sufficient in producing electrical power and generate 60% less carbon dioxide compared with other Tokyo Tech research buildings with a similar research environment and size,” explains Ihara. “We have achieved both goals, and thereby produced a model for constructing energy-efficient and environmentally friendly state-of-the-art buildings in the future.”



EEI

Some of the features of EEI include: 4,570 solar cell panels set according to unique design configurations to maximize electricity

generation—attached to the roof and southern- and western-facing walls—that have a power generation capacity of 650 kW in total (and can be tilted to allow natural illumination of the rooms and to use natural air flow to prevent a voltage drop due to temperature increases in the solar cells); a system to supply waste heat from a 100 kW fuel-cell system to outdoor air conditioners; motion sensors in the clean rooms that efficiently control the fan filter units and automatically close the doors of unused draft chambers; and a building constructed to withstand an earthquake of magnitude 6.0.

“Over 30% of the total power used for the building was generated by solar cells,” explains Ihara. “So we were able to be nearly self-sufficient by combining natural energy with stable power from fuel cells and an efficient, energy-saving system design. And, notably, we saved approximately \$300,000 in the cost of electricity, gas, and water between March 2012 and February 2013.”

Currently, Ihara is collaborating with software and data communication companies on the development of the “Ene-Swallow” smart grid to automate power management of the build-

ing. “We use the IEEE1888 international internet communication protocol to collect energy data from equipment and



Manabu Ihara

devices made by different manufacturers,” explains Ihara. “We have launched the Tokyo Tech ‘Green Hill Concept’ to manage the power to the facilities at our two other campuses at Tamachi and Suzukakedai. We expect our campuses to provide a proof-of-concept model for the city of the future.”

The “Ene-Swallow” smart grid not only offers an efficient means for power management, but will also enable an uninterrupted power supply using backup batteries during natural disasters such as earthquakes—a fact of life in Japan.

Tokyo Tech Environmental Energy Innovation Building:
www.titech.ac.jp/english/research/stories/eei_building.html



The University of Electro-Communications

Leading Research in Optics, Photonics, Wireless Communications, and Fuel Cells

The University of Electro-Communications (UEC) in Tokyo is a small, luminous university at the forefront of applied sciences, engineering, and technology research. Its roots go back to the Technical Institute for Wireless Communications, which was established in 1918 by the Wireless Association to train so-called wireless engineers in maritime communications in response to the Titanic disaster in 1912. In 1949, the UEC was established as a national university by the Japanese Ministry of Education, and moved in 1957 from Meguro to its current Chofu campus in Tokyo.

"Until a few years ago we taught courses on Morse code and radio navigation," explains **Wataru Mitsuhashi**, member of the board of directors responsible for research strategy at UEC. "The equipment we used for these courses is now on display in our Museum of Communications!" With approximately 4,000 stu-



Wataru Mitsuhashi

dents and 350 faculty, UEC is regarded as a small university, but with particular expertise in wireless communications, laser science, robotics, informatics, and material science, to name just a few.

The UEC was selected for the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Program for Promoting the Enhancement of Research Universities as a result of its strengths in three main areas: "optics and photonics research, where we are number one for the number of joint publications with foreign researchers; wireless communications, which reflects our roots; and materials-based research, particularly on fuel cells," explains Mitsuhashi.

UEC's main research reforms include the introduction of a new personnel salary system enabling the appointment of staff with a diverse range of experience; enhancement of its international standing by introducing an improved system to hire top-class researchers from all over the world; creation of an international photonics hub for laser and optics research; and training of university research administrators (URAs).

The University of Electro-Communications:
www.uec.ac.jp/eng

Institute for Laser Science

The Institute for Laser Science (ILS) is one of the most active research groups based in optics and photonics in Japan, with 50 UEC faculty members conducting research at the institute. "Our international approach to research has enabled us to make tremendous advances since the institute was established in 1978," says **Hitoki Yoneda**, director of ILS. "We have published the largest number of multi-institute international joint papers in Japan, which underscores the international nature of this institute. What is remarkable is that ILS has only eight full-time core research staff."

Major milestones since 1978 can be divided into six areas: development of a laser driver for inertial confinement fusion; atom optics, Bose-Einstein condensates, and cold atoms; ultra-short pulsed lasers and high-energy density science; highly charged ions; high-frequency

stabilized lasers and gravitational wave antennas; and quantum solids.

Findings from research conducted at ILS have often found further scientific application. One example is the high-quality optics and ultrahigh stability lasers that are being used for the construction of Japan's Kamioka Gravitational Wave Detector (KAGRA) to detect the gravitational waves predicted by Einstein's theory of general relativity. The project involves building two 3 km long laser interferometric gravitational wave detectors in a coal mine in Kamioka, Japan. Other "big-science" applications are highly stable lasers for precise frequency references for the X-ray free-electron laser, SCALA at the SPring-8 synchrotron in Japan, and the Atacama Large Millimeter/sub-millimeter Array (ALMA) telescope in Chile.

In 2000, ILS researchers reported the development of ceramic solid-state lasers, constructed from composite materials based on Cr⁴⁺-doped crystals (Yb:YAG/Cr⁴⁺:YAG) and capable of increased power output up to four orders of magnitude greater than current systems.

Progress has also been made in the application of highly charged ions (HCI) to fundamental science. In particular, ILS developed a greatly improved version of the electron beam ion trap for astrophysics, nuclear fusion, and ultrashort wavelength lithography. Notably, ILS research-

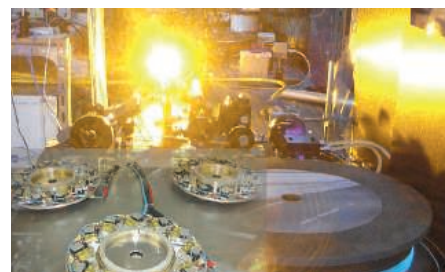
ers have demonstrated the use of HCI for forming a "hollow atom" in silicon and titanium oxide surfaces.

The research at ILS forms the basis for training and educating the next generation of scientists and engineers in this field. "We demonstrate unique experiments to students," says Yoneda. "For example, the management of sudden explosions and equipment failure are two daring experiments in our educational program. We also collaborate with partners overseas to organize regular courses on topics such as high-power lasers and their applications, and warm dense matter."

The outlook of postdoctoral fellows at ILS is truly global, with collaborations formed between ILS and the University of Southampton and the University of Edinburgh in the United Kingdom, Nanyang Technical



Hitoki Yoneda



High Power Fiber Lasers



University in Singapore, the University of Toronto in Canada, and Stanford University in the United States. "We also have strong historical links with scientists in Russia, in

particular, the Russian Academy of Sciences," explains Yoneda. "We plan to increase our international outreach by using the funding from the program to hire experts in optics and

photonics on a global scale."

Institute for Laser Science:
www.ils.uec.ac.jp

Advanced Wireless Communication Research Center

The Advanced Wireless Communication Research Center (AWCC) was established in April 2005 as a global research center for wireless communications and advanced wireless technology education based on both theory and practice. Although UEC was founded to lead Japan's wireless technology efforts following the Titanic accident, much has changed since then. "Our research reflects our commitment to an innovative and integrated approach for developing wireless technologies for the future," says **Yasushi Yamao**, director of AWCC.

AWCC also aims to transfer the fruits of its laboratory research to society via industry-focused technology transfer. "Currently, 36 of the 350 faculty members at the university are conducting research at AWCC," explains Yamao. "These include three full-time faculty, four concurrent professors, 20 research collaborators, and nine visiting professors. All have extensive links to industry in order to facilitate technology transfer."

With the mission of realizing ubiquitous and "cognitive" wireless communications, AWCC is pursuing the following projects:

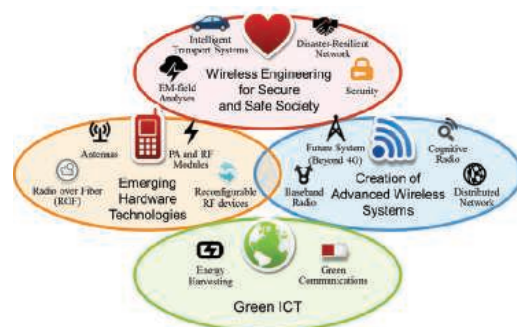
- Wireless engineering for a secure and safe society
- Emerging hardware technologies
- Green information communications technology
- Creation of advanced wireless systems.

Recent research findings include the development of a massive, wireless rewritable commercial price tag system incorporating 30,000 terminals. "This is an example of an industrial collaboration on ubiquitous wireless communications technology," says Yamao. "Our innovative protocol enables the connection of many devices as part of a low-power consumption, long-life, battery-operated system for use in large shops and supermarkets."

In response to demands for methods to monitor radiation levels in the wake of the Fukushima accident, AWCC has constructed an ad hoc wireless network of sensors to measure radiation levels. The system enables the measurement of temporal changes in radiation, including seasonal changes.



Yasushi Yamao



Areas of Research at AWCC

Advanced Wireless Communication Research Center:
www.awcc.uec.ac.jp/awcceng/index.htm

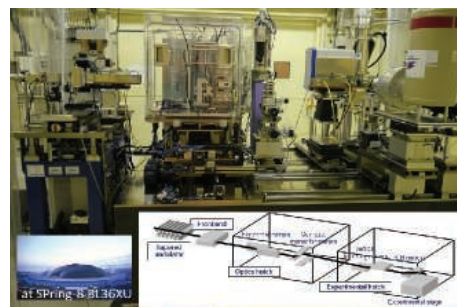
Innovation Research Center for Fuel Cells

Recent reports of cars powered by hydrogen fuel cells have captured the world's imagination with the hope of a clean energy source to mitigate environmental issues. The advent of commercial fuel cell automobiles is the result of intense basic research at many academic institutions. **Yasuhiro Iwasawa**, director of the Innovation Research Center for Fuel Cells, is one of the center's leading scientists and has made major contributions to unraveling the mysteries of the fundamental mechanisms underlying the generation of electricity from fuel cells. Iwasawa is the head of an approximately US\$26 million project to use X-ray absorption fine structure (XAFS) spectroscopy to probe

the atomic structure of catalysts in fuel cells, in situ and in real time. "Fuel cell catalysts are a black box right now," explains Iwasawa. "The atomic structure, reaction mechanisms, and degradation mechanism are still unclear. So we have built an 80 m beamline at the SPring-8 synchrotron facility in order to use XAFS to clarify these issues. This is the only XAFS beamline in the world capable of performing high-level in situ XAFS, real-time XAFS, and spatially resolved XAFS."

Specifically, Iwasawa and his colleagues are focusing on the XAFS analysis of the cathode catalyst inside the membrane electrode assembly during power generation. Fuel cell reactions are such swift, dynamic processes that high-spatial-resolution analysis is critical. "Our approach has several unique features," explains Iwasawa. "Being in situ and temporal, we can monitor chemical and structural changes down to a time scale of 100 μ s at 100 nm spatial resolution, and at 1 μ n 3-D resolution under the operating conditions used in our experiments."

Results from these unprecedented analyses at SPring-8 have yielded intriguing insights into



Time-Resolved and Spatially-Resolved XAFS Equipment at SPring-8 BL36XU Beamline

how catalysts behave. Notable findings include elucidation of the structural changes in the Au(core)-Pt(shell)/C catalyst during the on-off process of fuel cells, and the discovery that the reaction rate of the Pt₃Co/C catalyst is faster than Pt/C, and in particular, that the reformation of the Pt-Pt bonds and the dissociation of the Pt-O bonds are fast reactions. These results are expected to enable the design of highly efficient and robust fuel cells for the automobile industry.

Innovation Research Center for Fuel Cells:
www.icfc.uec.ac.jp



Yasuhiro Iwasawa

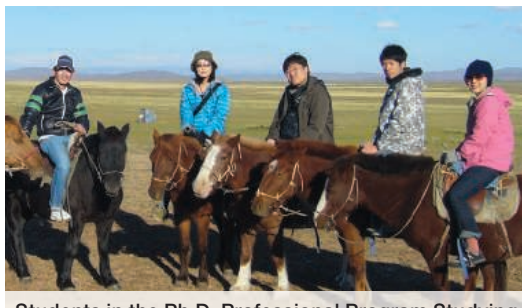


Nagoya University

Research and Education for Sustainable Global Development



Michinari Hamaguchi



Students in the Ph.D. Professional Program Studying in Mongolia



Toyoda Auditorium

Nagoya University was established in 1871 and is internationally recognized for its contributions to basic research including discoveries by its four Nobel Laureates: Ryoji Noyori (2001, chemistry), Osamu Shimomura (2008, chemistry), and Toshihide Maskawa and Makoto Kobayashi (who shared the 2008 prize in physics). Nagoya University is also the birthplace of the gallium nitride blue light-emitting diode, invented by Isamu Akasaki.

“From *Na-go-ya Dai-gaku* to Nagoya University’ is our adage, which captures the essence of our plans to globalize education and research,” says **Michinari Hamaguchi**, president of Nagoya University, as he sets out his plans to create an environment for young scientists to conduct cutting-edge research that contributes to the global society. “Here *Dai-gaku* is Japanese for university, and this dictum refers to our commitment to improve the global visibility of Nagoya University. The funding from the MEXT [Ministry of Education, Culture, Sports, Science and Technology] Program for Promoting the Enhancement of Research Universities will be used to initiate reforms to enhance research programs, such as projects at the new Nagoya University Institute of Transformative bio-Molecules [ITbM, see sidebar], which is one of nine World Premier Initiative [WPI] projects chosen by MEXT.”

Nagoya University’s research strategy is an integral part of a plan launched by President Hamaguchi in 2009, which includes improvements in internationalization and research through initiatives such as the G-30 Program and Super Global University Strategy as well as a program to specifically attract international students from Asia to study medicine and agriculture.

The G-30 Program offers English language courses and scholarships for approximately 50 international students annually. There are six courses and 11 programs for undergraduates and seven courses and 17 programs at the graduate school, all taught in English, in subjects ranging from engineering to economics. “We want to increase the number of international students from the current 2,200 [15% of the total number of students] to 3,000 [20%] by 2020,”

says **Hideyo Kunieda**, trustee and vice president for research and student support. “We also want to increase the number of Japanese students going overseas, from what is currently around 600, to 1,000—out of a total of approximately 2,200 students in one grade studying at Nagoya University—by 2020.”

It is hoped that the Super Global University Strategy will support the establishment of classes at centers set up by Nagoya University in other countries. One example is the Center for Asian Legal Exchange (CALE), an umbrella organization managing the university’s Education and Research Centers for Japanese Law. The five centers, offering courses in Japanese law and language, are located in Uzbekistan, Mongolia, Vietnam, Cambodia, and Myanmar, with plans to open new centers in Indonesia and Laos. “We want to nurture people to devise laws applicable throughout Asia, similar to the laws governing the European Union,” explains Hamaguchi. “Graduates from the courses given at CALE go on to influential positions in government and academia. They are also an important part of the Nagoya University’s international network.” Other similar centers have already been established in Germany, China, and the United States.

The university also invites international students from Asia to courses at its Nagoya campus in subjects related to medicine and agriculture. An example is the Young Leaders Program in Healthcare Administration, a one-year Master’s course that engages students from 14 countries in Asia and Eastern Europe. Other facilities for education include the Endoscope Training Center located in hospitals in Vietnam, and overseas centers such as the Technology Partnership of Nagoya University, Inc. (NU Tech) in North

Carolina in the United States, and the “Europe Center” located in Freiburg, Germany.

One of the more recently established programs is the Women’s Leaders Program to Promote Well-Being in Asia, which was granted support in 2013 by the MEXT/JSPS (Japan Society for the Promotion of Science) under the Program for Leading Graduate Schools.

Students at Nagoya University can also take courses offered by the joint degree program in medical sciences





with the University of Adelaide in Australia, the joint educational program with University of Freiburg and University of Strasbourg, and the Japan-U.S. Advanced Collaborative Education Program (JUACEP) with the University of Michigan and the University of California, Los Angeles.

STRATEGIES AND GOALS

“We have introduced a new incentive system to encourage our young researchers, taking them from a position of being a source of ‘labor’ to one of ‘leaders,’” explains Hamaguchi. “For instance, we will increase the salaries of researchers who acquire funding from competitive grants such as the WPI project.” Another important set of reforms are related to assignment of tenured faculty positions. Hamaguchi stresses the need for transparency and accountability in the process of appointing faculty positions. “All assistant professor positions will be part of our tenure-track system with evaluation within five years, after which tenured lecturer posts will be offered to those who meet our requirements,” explains Hamaguchi.

“It is essential to create an environment for young scientists to conduct interdisciplinary research that contributes to global sustainability,” adds Hamaguchi. “One of the most important aspects of the reforms is changing the mind-set of staff—both faculty and administration—to recognize the importance of the changes in the creation of an internationally competitive university.”



To achieve these goals Nagoya University has integrated previous resources such as the Office for Initiatives for Industry, Academia and Government Cooperation (which includes the Technology Licensing Office) and the University Research Administration (URA) Office to form the Science and Industry, Academia and Government Cooperation Center, led by the university's Vice President Hideyo Kunieda. “Strengthening the gathering, analysis, and dissemination of information, enhancing the ability to initiate new projects, and having a single, unified system for interacting with external organizations are the central functions of the new research management system,” explains Kunieda. To support this, the number of URAs will increase from the present 34 to 44.

Hamaguchi notes that the program will also be an important means for increasing the number of tenured faculty members under the age of 35. “Japanese national universities all share this problem,” explains Hamaguchi. “Young scientists are often employed on short-term postdoctoral contracts, a situation that does not enable them to conduct the independent research necessary for tenured positions. The program aims to alleviate this situation.”

Nagoya University:
en.nagoya-u.ac.jp

Institute of Transformative bio-Molecules

Kenichiro Itami, director of the Nagoya University Institute of Transformative bio-Molecules (ITbM), wants to change the world. “At ITbM, we want to design molecules to solve global problems related to the environment, food production, and medicine,” he explains. “Our international teams of chemists and biologists are creating unique bioactive molecules with highly specific and targeted functions with the goal of addressing the most daunting problems facing society.”

ITbM is a 10-year project with annual funding of US\$5 million. It was launched in April 2013 after being selected by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) as one of the projects in WPI. Researchers at ITbM are undertaking three major projects: control of biological systems, visualization of biological systems, and synthesis of novel biofunctional molecules.

“Approximately 55% of our research staff are from overseas,” says Itami. “We have excellent bilingual research support staff, including

our ‘WPI-Mother’ who plays a central role in looking after the daily needs of our overseas researchers both inside and outside the lab.”

The “mixed-labs” concept is an important feature of the research infrastructure of ITbM, where chemists, biologists, and theoreticians share the same laboratory space. “This under-one-roof approach is the driving force for our research,” says Itami.

The ITbM has 10 principle investigators—seven based at Nagoya University and three overseas at ETH-Zurich in Switzerland, Queen's University in Canada, and the University of Washington in the United States. Researchers at ITbM also collaborate with the National Science Foundation's Center for Selective C-H Functionalization and RIKEN's Center for Sustainable Resource Science. In addition to international collaboration, ITbM has also established three important centers at Nagoya University that are uniquely geared toward research. These are the Molecular Structure Center, the Chemical Library Center, and the



Kenichiro Itami

Live-Imaging Center.

“We are mixing synthetic chemistry with systems biology to create new synthetic paradigms,” says Itami. “We plan to create our designer molecules both in beakers and in living organisms.”

Institute of Transformative bio-Molecules:
www.itbm.nagoya-u.ac.jp



Toyohashi University of Technology

“Value Creation Engineering” for New Industries



President Yoshiyuki Sakaki



Vice President Makoto Ishida



Toyohashi Tech Campus Buildings

“Our research and education has been recognized through the receipt of three major project awards this year,” explains **Yoshiyuki Sakaki**, president of Toyohashi University of Technology (Toyohashi Tech). Sakaki is a molecular biologist who was chosen as a Person of Cultural Merit in 2013 by the Japanese government. The three projects are: the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Program for Promoting the Enhancement of Research Universities; the Program for Leading Graduate Schools on Brain Information Architects; and a joint project to establish an overseas campus in Penang, Malaysia.

Toyohashi Tech was established approximately 38 years ago. With about 2,000 students and 200 faculty members, it is one of the smallest national universities in Japan. “A little giant, comparable in size to Caltech in the USA,” says Sakaki. “Being selected for the MEXT program is a like a shot of adrenaline for our researchers; we’re very excited about it!”

Sakaki notes that the selection procedure for the program was top-down and based on independent objective metrics, such as citations, royalties from patents, and research funding grants received. “Toyohashi Tech was selected as one of 22 outstanding research universities and research institutes,” continues Sakaki. “This acknowledges our research to date, and the contributions of the Electronics-Inspired Interdisciplinary Research Institute [EIIRIS], the engine driving research at Toyohashi Tech” (see sidebar below).

OBJECTIVES OF THE PROGRAM

“In our projects we will devise methods for creating positive value from nominally negative assets,” explains **Makoto Ishida**, vice president of Toyohashi Tech and the person in charge of research. “For example, using waste to create valuable assets, such as biofuels, and extending the functions of our imaging devices to universal self-diagnosis health care biosensors. We refer to this as Value Creation Engineering.”

The Value Creation Engineering project will be managed by the Research Administration Center—set up in December 2013—which includes the University Research Administration Office.

The creation of new industries is one of the major objectives of the Toyohashi Value Creation Engineering project. “Our university has an excellent record in technology transfer,” explains Ishida. “We want to build on this by linking up with global partners to generate ideas for new innovative industries.” Toyohashi Tech will use its expertise in interdisciplinary research, together with research talent at EIIRIS, to achieve these goals. The project will entail collaboration with research institutes and companies worldwide, hiring research staff and students from overseas, and reforms in the personnel system.

Toyohashi University of Technology:
www.tut.ac.jp/english

The Electronics-Inspired Interdisciplinary Research Institute

EIIRIS is the flagship research hub of Toyohashi University of Technology. It was established in October 2010 to build on Toyohashi Tech’s expertise in microelectronics and act as a platform for the creation of new research paradigms combining electronics with the life sciences. The institute aims to solve problems in diverse areas including environment, energy, food production, and population. For outreach, EIIRIS organizes international conferences, such as The Irago Conference Series, and advertises its activities through regular press releases and a

quarterly Toyohashi Tech e-newsletter.

Research covered at EIIRIS includes advanced medical technology, brain-related technology, and green technology. EIIRIS has 11 researchers, two technical assistants, two research support staff employed directly with EIIRIS funding, and 10 tenure-track researchers.

EIIRIS has a range of high-quality research facilities. On the first floor of EIIRIS-1 there is a 1,500 m² clean facility, completed in 2010. It houses lithography process rooms, Raman spectroscopy and scanning electron

microscopes, and optics areas with advanced fluorescent microscopes and systems for measuring minute electrical signals used in neuroscience research. EIIRIS-2 is a huge 2,300 m² clean facility connected to EIIRIS-1 via a bridge on the third floor. It contains design and fabrication equipment for large-scale integrated circuits and micro-electro-mechanical systems (MEMS). Finally, EIIRIS-3 is a life sciences experimental facility for full-scale experiments using animals—an unusual research facility for an engineering university.

One highlight of research at EIIRIS is



Electronics-Inspired Interdisciplinary Research Institute

Researcher Spotlight

Kazuaki Sawada

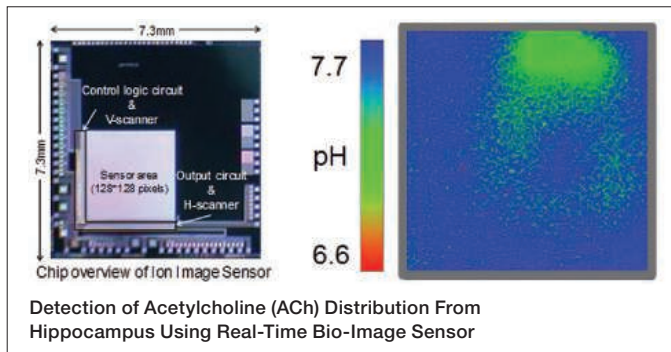
has integrated conventional biosensing technology, such as functionalized membranes, with charge-coupled device (CCD) image sensors to produce an innovative camera for monitoring the two-dimensional (2-D) movement of compounds on a microscopic scale. The device consists of an array of CCDs covered with a functionalized membrane. The 2-D variation in the ion concentration leads to charge accumulation in the CCD devices, which is presented visually as color-coded images. "The initial device was used for 2-D imaging of the pH outside cells," says Sawada. "We have recently extended the applications of the sensor for monitoring neurotransmitters in cells. This signal can be connected to external circuits for applications such as a chemical-human machine interface to monitor and control chemical reactions in the body in real time via an implanted device."

Sawada has used the ion imaging sensor for the first ever, real-time imaging of acetylcholine (ACh). Knowledge of the variations of the concentration of ACh may yield clues for the treatment of diseases such as Alzheimer's.

Sawada Laboratory: int.ee.tut.ac.jp/icg

Shigeki Nakauchi studies visual perception and cognition to answer questions such as: Why are humans able to recognize objects the instant they see them but machines cannot? His research covers three main areas: understanding visual systems, measuring brain activity by electroencephalography, and visualizing otherwise invisible information using infrared-spectrum imaging. A particularly intriguing set of experiments involves studying the differences in the perceptual processing abilities of experts and novices. As an example, Nakauchi

the so-called Toyohashi Probe, invented by **Makoto Ishida** and his colleagues. The probe consists of vertical silicon nanowire electrodes—produced by a vapor-liquid-solid synthesis process—that are inserted into living tissue to detect electrical signals in cells. EIIIRIS researchers **Tetsuhiro Harimoto** and Hideo Oi are developing arrays of multichannel electrodes that they plan to use to measure neural signals in the retina, as well as in a brain-machine interface device.



has been investigating the relationship between the subjective quality and pictorial features of a pearl, as seen by a pearl appraiser and a novice. "I am developing an optical system to capture how an expert sees features," says Nakauchi. "This research is ultimately aimed at elucidating neuromechanisms of congenital ability and memory."

Nakauchi Laboratory: www.vpac.cs.tut.ac.jp

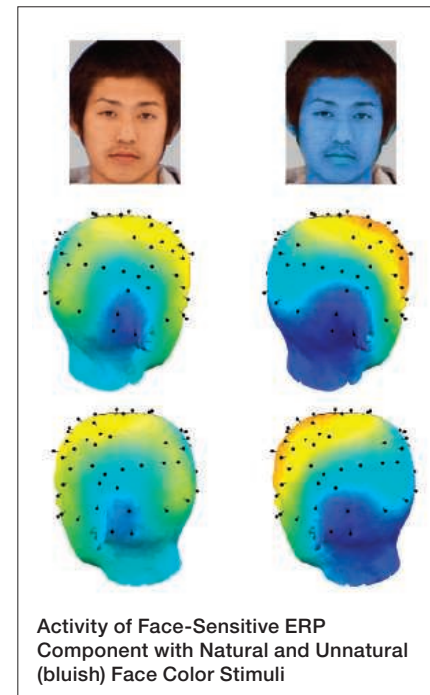
Hiroyuki Daimon is studying the efficient utilization of biomass, carbon dioxide (CO_2), and heat as part of a major project on creating sustainable, recyclable energy to counter the adverse effects of climate change. "We have constructed a prototype within the Toyogawa sewage treatment plant," says Daimon. "We collect biomass, ferment it to produce methane, and use the methane gas to generate electricity. The residue is used to produce fertilizer."

Notably, Daimon and his research group leave nothing to waste. Any CO_2 produced during the fermentation is introduced into a marine plant farm for seaweed production, and the CO_2 and heat generated during the generation of electricity is delivered into a greenhouse for growing tomatoes. "These are early demonstration experiments with which we have begun the Toyogawa Biomass Park," says Daimon. "We invite visitors to join us and even taste our delicious tomatoes and vegetables!"

Daimon Laboratory: water.ens.tut.ac.jp/index.html

Other exciting research, in this case in the field of functional materials, is being done by EIIIRIS scientist **Tran Viet Thu** and colleagues who have synthesized graphene and composites by bacteria-mediated reduction, and silver-reduced graphene oxide nanohybrids for the development of highly efficient catalysts.

Electronics-Inspired Interdisciplinary Research Institute: www.eiiris.tut.ac.jp



Toyogawa Biomass Park Demonstration Facilities at Toyogawa Sewage Treatment Plant





Kyoto University

Applying Globally Minded Academic Freedom to Multidisciplinary Research



Hiroshi Matsumoto



Kyoto University Clock Tower



Self Reliance and Self Respect

Kyoto University was established in 1897 as Japan's second Imperial University, responding to calls from Kyoto residents for the creation of educational and research facilities in the city (which was the capital of Japan from 794 to 1868).

"The people of Kyoto have tremendous respect for scholars and scholarship," explains **Hiroshi Matsumoto**, president of Kyoto University. "The unique combination of the rich culture and history of Kyoto and the university's commitment to globally minded academic freedom and an integrated, multidisciplinary approach to research has spawned discoveries by Kyoto University researchers that have changed the world. Their work exemplifies our motto, 'self reliance and self respect.'"

Kyoto University's prowess as a research powerhouse is evidenced by the major scientific breakthroughs made by the eight Nobel Laureates affiliated with the university. The most recent Nobel winner is **Shinya Yamanaka**, director of the Center for iPS Cell Research and Application (CiRA), who shared the 2012 prize for physiology or medicine for his work on reprogramming mature cells to become stem cells. Furthermore, in the field of mathematics, two of the three Japanese recipients of the Fields Medal—formally known as the International Medal for Outstanding Discoveries in Mathematics and widely referred to as the "Nobel Prize for mathematics"—are Kyoto University alumni. Kyoto University plans to reform its research activities by creating an environment where knowledge can extend beyond existing boundaries. This borderless pool of knowledge will be implemented by promoting internationalization, employing staff with diverse expertise and experience, investigating unexplored disciplines and topics on the fringes of what is currently accepted as science, spurring invention and innovation to connect academic curiosity with the needs of society, and creating a sustainable management system to overcome organizational and institutional barriers.

"Our research plans include two unique ideas reflecting the university's commitment to nurturing innovative solutions to tackle global issues such as climate change," says Matsumoto. "The first is known as *mikagaku* in Japanese, which literally translated means 'proto-science.' Here we are exploring the

unexplored and in some cases, the unimagined. These are ideas and concepts that are yet to be recognized as scientific disciplines. An example of *mikagaku* is research on induced pluripotent stem cells—the notion of reprogramming adult cells was unimaginable until the pioneering work by Shinya Yamanaka."

The other Kyoto-style approach to research is embodied in the Hakubi Project, launched in 2009. "Each year we select 20 young scientists and give them five years to pursue research on a topic of their choice, at any location in the world," explains Matsumoto. "They receive excellent funding and administrative support, with only the bare minimum of oversight. It's a very popular project. This year we have had 644 applications—including 236 from overseas—for the 20 posts. This project is an example of our multidisciplinary and international approach to addressing multifaceted global issues."

One of the ongoing Hakubi Projects involves scientists devising a system for giving health checks to the elderly in the Kingdom of Bhutan and the development of a microscope that can image a DNA double helix in water.

Kyoto University will launch many projects as part of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Program for Promoting the Enhancement of Research Universities. One such project is the Supporting Program for Interaction-based Initiative Team Studies (SPIRITS), where the university will support the launch of 100 new projects over 10 years, irrespective of discipline.

There will also be a new and renewed John Mung Program to support international development for university members. The program provides assistance for faculty members to conduct



Kiyoshi Yoshikawa

their research abroad, for administration staff to gain experience in international affairs, for university research administrators (URAs) to develop their research promotion skills, and for students to study overseas. (The title of this program is the English name of the Japanese sailor, Nakahama Manjiro, who, after being rescued from a shipwreck by an American whaler, spent a number of years studying abroad and later returned to become a leading interpreter of Western culture during the Meiji era.)



Another major project, called *Hyakkasōmei*, creates a platform for scholars from different countries and disciplines to gather and freely discuss issues that may lead to new, as yet unrecognized scientific disciplines encompassed in the concept of *mikagaku*. “Kyoto University is famous for its open-minded research culture,” says **Kiyoshi Yoshikawa**, executive vice president for research.

The university will also establish an exploratory initiative institute as a platform to test new reforms, such as rewarding the performance of outstanding researchers with increases in salary. If the reforms prove to be successful, they will be applied throughout the university.

“Resources from the MEXT program will be used to hire more URAs to further facilitate creative research by reducing the administrative workload of principal investigators,” adds Yoshikawa. The university will hire 20 new URAs in addition to the current 30 URAs to manage the new initiatives. It will also launch the Kyoto University Skill Development Program as a career development path for URAs.

“The URAs will fulfill many roles, including working with the directors of the university to analyze research trends and project them into the future, and to propose new areas of research to pursue,” says **Koji Tanaka**, director of the Kyoto University Research Administration Office.

INTERNATIONAL OUTREACH AND DEVELOPMENT

Kyoto University maintains an extensive global network to pursue its education and research via collaborative programs, joint international symposia, and exchange agreements. Notably, in the 2011–2012 academic year, approximately 3,000 international researchers worked at

Kyoto University while approximately 8,100 Kyoto researchers worked overseas for differing periods of time during the year.

Collaboration in Asia includes the Kyoto University Bhutan Friendship Program and the Japan International Cooperation Agency on supporting engineering in Myanmar. The university maintains many similar activities around the world. “The 1st Bristol-Kyoto Symposium held in Bristol in January 2013 is an example of the university’s commitment to fostering strong international relationships,” says Yoshikawa. “Approximately 90 people from Kyoto traveled to Bristol for face-to-face discussions on enhancing existing research collaborations and initiating new ones.” The 2nd Bristol-Kyoto Symposium was held a year later in Kyoto in January 2014. Other such meetings were held with three institutions in Switzerland in November 2013, and with National Taiwan University in December 2013.

The 2x by 2020 (Double by Twenty-Two) Initiative, formulated under the leadership of **Michiaki Mishima**, executive vice-president for international affairs and hospital administration, is the roadmap for Kyoto University’s international research strategy until the year 2020. The plan includes bold and

ambitious targets such as more than doubling the number of international researchers and faculty members from 3,190 to 6,500 and the number of international students from 2,082 to 4,300.

Even more, Kyoto University has set a goal to become one of the top 10 universities in the world, as ranked by the Times Higher Education World University Ranking, by 2020.



Koji Tanaka

Kyoto University:
www.kyoto-u.ac.jp/en

Researcher Spotlight

Aya Yanagawa is an assistant professor in the Laboratory of Innovative Humano-Habitability in the Institute of Sustainable Humanosphere, Kyoto University. “I decided to pursue a career in research because I am fascinated by the hidden beauty and strategies for survival in all living things,” she says, speaking from CNRS, Laboratoire Évolution, Génomes et Spéciation, in Paris, where she is completing a nine-month sabbatical sponsored by Kyoto University.



Aya Yanagawa

Yanagawa is currently studying the grooming behavior in insects for removing pathogens and microbes from the surfaces of their bodies. “I am investigating insect-pathogen relationships in termites and fruit flies, that is, the links that may exist between behavior and disease defense mechanisms.”

Specifically, Yanagawa is looking for links between insect pathology, microbial control, and the behavioral resistance of insects against microbial infection. “Generous funding by Kyoto University has enabled me to study the insect hygiene behavior of termites in Japan and to travel to France to look at flies,” she explains. “The university provides an excellent environment for young researchers.”

In the long term, her research could lead to the development of ecologically friendly, biological pest controls as alternatives to chemical insecticides. “The world is facing a plethora of daunting problems, such as pollution, energy shortages, and agricultural issues such as food security,” says Yanagawa.

“I believe that the future of agriculture will be brightened by employing integrated pest management techniques. As a researcher in agriculture, I hope that my study will contribute to a more harmonious co-existence between natural systems and human activity.”

Yanagawa adds that her wishes may now be one step closer to becoming reality, as recent research has revealed that behavioral resistance is one of the most effective means of fighting pathogenic infection in insects. Her project should yield clues for finding effective ways of using pathogens in biological control. Furthermore, investigating the behavior of the fruit fly, *Drosophila melanogaster*, could lead to the discovery of new genetic information about disease transmission by flies, which will be important in medical entomology, hygiene, and sanitation.

Laboratory of Innovative Humano-Habitability, Institute of Sustainable Humanosphere:
www.rish.kyoto-u.ac.jp/English/members-en.html

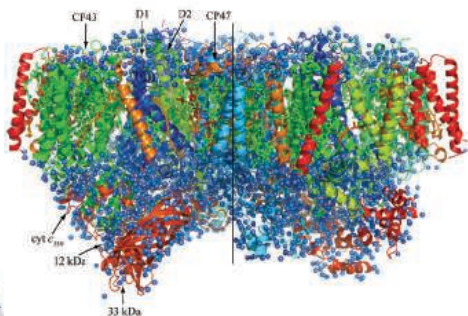


Okayama University

A Multifaceted Center for Interdisciplinary Research



Vice President
Shin-ichi Yamamoto



Protein Structure of Photosystem II



Catalyst for Water Splitting



Okayama University Library

Okayama University is a comprehensive institution with approximately 1,300 faculty and 14,000 students. It offers courses in subjects ranging from medicine and pharmacy to humanities and physical sciences and is situated approximately three hours west of Tokyo by high-speed rail. The roots of the university go back to the Medical Training Place set up in 1870 with the support of the lord of Okayama.

“An independent report compiled by the National Institute of Science and Technology Policy shows our research strengths in physics and basic life sciences,” says **Shin-ichi Yamamoto**, executive director and vice president for research. “As part of the Ministry of Education, Culture, Sports, Science and Technology [MEXT] Program for Promoting the Enhancement of Research Universities, we have established the provisionally named Organization for Global Advanced Interdisciplinary Sciences, which will implement reforms that build on our strengths to create a globally competitive, research-based university.”

The main pillars of the Okayama University’s overall research strategy are the Center of Innovation project, enhancing translational clinical research at Okayama University Hospital (a hospital that was selected as one of the 15 core hospitals for clinical research in Japan), and the abovementioned MEXT program.

“The Organization for Global Advanced Interdisciplinary Science will coordinate our research strategy by collaborating

with other institutes located near Okayama, such as RIKEN’s SPring-8 synchrotron, the X-ray free electron laser (SACLA) in Harima, and the K-supercomputer in Kobe,” explains Yamamoto.

Toru Numaguchi was appointed senior research administrator in 2012. “Some of the major challenges in the implementation of research reforms will be defining new areas of research over the next, say, 10 years, as well as initiating productive and high-quality international research, and estimating funding requirements over that 10-year span,” says Numaguchi.

The directors of research at Okayama University have used and will continue to use powerful databases to conduct bibliometric studies in order to benchmark research, find partners for research and technology transfer, and develop future strategies.

“Data analysis and surveys will become increasingly important for devising research strategies at universities in Japan,” says Yamamoto. “Data from this type of benchmarking may play a greater role in decisions for funding major projects, as in the case of the MEXT program, which was a top-down decision based on independent performance data.”

Some of the centers at the university benefiting from the MEXT program are highlighted in the sidebars below.

Okayama University:

www.okayama-u.ac.jp/index_e.html

Creating New Materials for a Green Future

“The Research Center of New Functional Materials for Energy Production, Storage and Transport was launched in July 2010,” says **Yoshihiro Kubozono**, director of the center. “It is the hub of an international and interdisciplinary research project based on innovative functional materials to develop science and technology for a sustainable, low-carbon society.”

Research at the center covers a broad range of areas:

- High-performance and high-efficiency

organic solar cell alternatives to the conventional photovoltaic devices, fabricated using inorganic materials such as amorphous silicon and other such semiconductors

- Development of novel solar cells based on new dielectric materials
- Development of low-energy consumption processes to manufacture high-performance organic field-effect transistors
- Synthesis of new nanomaterials for storing hydrogen and methane
- Development of new superconducting

organic materials

- Development of high-temperature superconductors using new design approaches
- Conversion of optical energy to electrical energy using processes based on biological systems.

“We are global in our approach to pursuing our goals,” explains Kubozono. “One of our colleagues from Okayama University is on a long-term stay at the University of Durham in the United Kingdom, where he is investigating new carbon-based superconductors.” Funding



from the MEXT program will be used to strengthen the university's global presence by inviting scientists from overseas to conduct research at the center.

Okayama University scientists are strongly encouraged to share their findings with the broader scientific community. "We regularly publish our findings in high impact journals, reflecting the high quality of our research," says Kubozono. "Notably, we have published a paper in the journal *Nature* each year since the launch of the center."

Kubozono was recently in the spotlight following the generation of potassium-doped



Yoshihiro Kubozono

picene, a semiconducting solid hydrocarbon. The material exhibits superconductivity properties at relatively high temperatures (up to 18 Kelvin, or -255.2°C). "Superconductivity

in carbon materials may be a new way to produce superconducting circuits for ultra-low power electronics," says Kubozono. "This is an excellent example of our focus on interdisciplinary science, with chemists contributing to physics."

Kubozono Laboratory:
interfa.rlss.okayama-u.ac.jp/index.html

Research Center of New Functional Materials for Energy Production, Storage and Transport:
www.science.okayama-u.ac.jp/RCNFM/indexeng.html

Photosynthesis Research Center

The Photosynthesis Research Center was launched in April 2013. "Our goals are to clarify the biochemical mechanisms of photosynthesis, in particular reactions related to light-induced water-splitting," explains **Jian-Ren Shen**, director of the center. "Our findings may enable the synthesis of catalysts for artificial photosynthesis—for a potentially unlimited source of clean energy."

The importance of Shen's research into the mechanisms of light-induced water-splitting led to its selection as one of the 10 Breakthroughs of the Year in 2011 published by *Science*. It was also awarded the prestigious 2012 Asahi Prize for outstanding accomplishments in the fields of academics and arts.

"The results are described in a 2011 *Nature* paper and are the culmination of 21 years of my research on photosynthesis resulting in the synthesis of ultra-pure, single-crystals of the so-called Photosystem II membrane protein complex, or PS II," explains Shen.

Ultrahigh resolution X-ray diffraction experiments on the PS II crystals conducted at the SPring-8 facility showed that it has a cubic-core of four manganese atoms, five oxygen atoms, and a calcium atom. This cluster of Mn_4CaO_5 catalyzes the light-induced splitting of water. "The distorted chair-like structure of the cluster proved to be difficult to synthesize artificially," says Shen. "But there is considerable industrial interest in doing so.

Success may yield a means of extracting clean energy from the sun."

Okayama University Photosynthesis Research Center:
www.okayama-u.ac.jp/en/tp/news/news_id2402.html



Jian-Ren Shen

Research Core for the Extreme Quantum World

Photons are the most abundant particles in the universe at about 410 particles per cubic centimeter, while the second most abundant are neutrinos (330 particles per cm^3). The physics of photons (light) is well established, but quantitative information about neutrinos, such as their absolute mass, is lacking despite their importance for explaining the structure and origins of the universe.

The physical properties of neutrinos are not well understood in large part because they are extremely difficult to detect. Current state-of-the-art research on neutrinos is expensive, time-consuming, and requires the use of massive particle accelerators and/or detectors buried deep in the Earth.

Now, **Noboru Sasao** and colleagues at the Okayama University Research Core for the Extreme Quantum World are proposing to conduct research in ordinary, inexpensive university labs in an attempt to demystify the world of neutrinos and the origins of the universe through the Spectroscopy with the Atomic Neutrino (SPAN) project.

"In SPAN, we are investigating the properties of neutrinos by analyzing the response of target atoms to incident laser light," says Sasao, leader of the SPAN project. "Our approach offers a relatively inexpensive and systematic method for obtaining preliminary data about neutrinos that could be used as the basis for more advanced and quantitative research on the origins of matter in the universe."

In these experiments, the researchers excite atoms or molecules coherently with laser light and detect and measure the energies of photons emitted due to the de-excitation, a

process called radiative emission of a neutrino pair (RENP). Neutrinos are impossible to detect directly but information about their absolute mass can be ascertained by analysis of the photon spectra. However, the rate of emission of light in the RENP process is extremely small, which in turn limits the probability of finding information about neutrinos in the emission spectra. "We intend to overcome this problem by using lasers to amplify the optical signal from samples," explains Sasao. "We refer to this amplification mechanism as macro-coherent amplification, and it manifests itself as two photons being emitted in the de-excitation process, which we have called paired super-radiance." Initial proof-of-principle experiments using a solid sample of parahydrogen are promising, showing a coherence time of longer than 20 ns. This is very long for solid materials, which typically have coherence times of less than 1 ns.



Noboru Sasao

Research Core for the Extreme Quantum World:
www.xqw.okayama-u.ac.jp/index.php

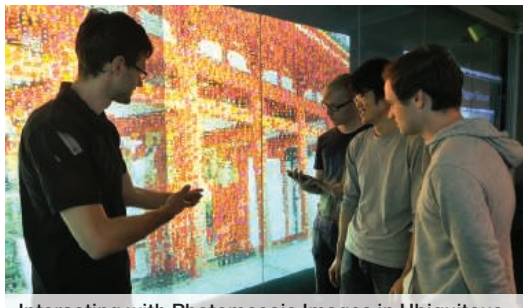


Nara Institute of Science and Technology

Young and Nimble: Big Achievements From a Small Research Center



Naotake Ogasawara



Interacting with Photomosaic Images in Ubiquitous Display Environment



NAIST Gate

Nara Institute of Science and Technology (NAIST) is one of Japan's youngest research-based institutes. Located in the Kansai Science City at the border of Nara, Osaka, and Kyoto, NAIST consists of three graduate schools: Information Science, Biological Sciences, and Materials Science. The faculty members are young—around 40% are under 39 years old—and the students international, with 30% of doctoral students from outside Japan.

"NAIST only offers graduate school courses," explains President **Naotake Ogasawara**. "We had our first intake of students 21 years ago and currently have approximately 200 faculty and 1,000 students, of whom 300 are pursuing doctoral degrees. NAIST researchers publish an average of 340 papers per year of which more than 40 are in the top 10% in their field according to international benchmarking data."

NAIST was established in 1991 with the intention of removing departmental barriers and promoting interdisciplinary research. This openness and flexibility have led to important discoveries by prominent scientists who have conducted research at NAIST. Examples include **Shinya Yamanaka**, who was awarded the 2012 Nobel Prize in physiology or medicine for his seminal work on reprogramming mature cells to restore pluripotency, work he began during his time at NAIST as a young researcher. Another high-profile NAIST researcher is **Ko Shimamoto**, who is internationally recognized for his pioneering work as a researcher on the hormone florigen and its role in the flowering of plants. "To further develop and extend these powerful research activities to the next generation, we always make efforts to discover and encourage the growth of young faculty members," says Ogasawara.

As part of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Program for Promoting the Enhancement of Research Universities, NAIST will launch the following initiatives: an international tenure-track program that will employ six young scientists as associate professors, with the prospect of tenure upon completion; annual travel support for up to five young faculty members to work overseas for approximately one year on collaborative research projects; one or more satellite research hubs will be set up overseas to conduct joint international research and establish an international research network for NAIST; and, in cooperation with overseas partners, NAIST will host one or more satellite laboratories that will be used as a base by scientists from overseas when they stay at NAIST.

"We are planning to employ six university research administrators [URAs] to manage this project based upon the findings of their ongoing institutional research," says **Naokazu Yokoya**, executive director and vice president. "They will act as a bridge between the researchers and executive directors of the university, introducing and promoting research from both academia and industry around the globe."

Kenji Kohno is the director of the Center for Frontier Science and Technology and one of the NAIST faculty members responsible for implementing the MEXT program. "The fact that we do not have an undergraduate school means that we do not show up in international university ranking data," says Kohno. "The current methods used for assessing universities do not accurately cover small, graduate schools like NAIST—so while we produce world-class research and researchers that are active in various fields around the globe, these are not clearly represented by this ranking methodology." New research currently attracting attention includes work on optical media interfaces headed by **Yasuhiro Mukaigawa**, investigations into the principles of plant development headed by **Keiji Nakajima**, and **Masakazu Nakamura's** research on organic compounds used to produce environment-friendly, low-energy electronic devices. Research highlights of these three young faculty members, underscoring the open and flexible environment at NAIST, are outlined on the following page.

Nara Institute of Science and Technology:
www.naist.jp/en/



Recording Output of Neural Circuit after Manipulating Input Using Light



Making the Invisible, Visible

Rays of light contain valuable information about the texture and shape of objects ranging from a bowl of fruit on a kitchen table to human skin or solutions of colloids. The nature of light rays is changed by reflection and scattering by objects before the light enters our eyes or is captured by a camera. Furthermore, in translucent materials it is not possible to directly measure the behavior of light as it is scattered within the material. A deep understanding of how light behaves and

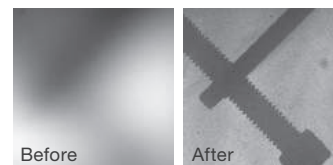


Yasuhiro Mukaigawa

is propagated in scattering media is important in many areas of research including image rendering, medical imaging, and materials characterization.

“My research activities are focused on optical media interfaces,” says **Yasuhiro Mukaigawa**, professor in the Graduate School of Information Science. “We use a wide variety of special cameras and image capture technologies to take photographs of objects, mathematically analyze the images, and reproduce complementary images of the same scene, which show features that are not visible to the human eye prior to analysis.”

An example of Mukaigawa’s research is the Parallel High-Frequency Illumination (PHFI)



Example of Using PHFI To Remove Scattered Light From a Cloudy Solution

that removes all scattered light emanating from an object, yielding clear and sharp images of objects inside cloudy liquids. “Objects inside a turbid solution are clearly delineated by this apparatus,” says Mukaigawa. “It makes the otherwise invisible, visible.”

Mukaigawa Laboratory:
omilab.naist.jp

Demystifying Plant Development and Cellular Signaling

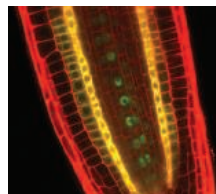
Examination of the cross-sections of plant leaves and roots under a microscope reveals beautiful patterns of intricately interweaving cells of myriad sizes and shapes, all arising from a lone zygote. “The question arises as to how these complex plant structures are derived from a single cell,” says **Keiji Nakajima**, associate professor in the Graduate School of Biological Sciences. “Our research aims to identify the basic principles of plant development using model plant species.”

Specific targets include studying the inter-cellular signal transduction pathways underlying the pattern formation of roots and embryos, and cell reprogramming triggered by embryogenesis. In this vein, the laboratory also studies

cell-cell communication in tissue patterning, where recent experiments have revealed novel signaling pathways enabling regulatory molecules such as transcription factors and microRNAs to travel between cells. Nakajima says that his group is now “focusing on molecular



Keiji Nakajima



Gene Expression Patterns in Plant Root

mechanisms enabling microRNAs to move from cell to cell and the evolution of such pathways.”

Another fascinating research area focuses on understanding cell reprogramming and pattern formation during embryogenesis, that is, the process of initiation and development of a plant embryo from a zygote. “We have discovered a key reprogramming factor in *Arabidopsis*—a small flowering plant we use as a model. We are currently working on its mechanism of action,” explains Nakajima. “And we are developing methods that utilize this reprogramming factor to propagate useful plant lines.”

Nakajima Laboratory:
bsw3.naist.jp/nakajima/English/

Organic Materials for Printable Devices

Masakazu Nakamura was hired by the Graduate School of Materials Science as a tenure-track professor, part of a program designed to promote young NAIST faculty members. He uses organic compounds to fabricate field-effect transistors, energy harvesting devices, and terahertz sensors. The aim is to produce low-energy, environmentally friendly electronics devices that can be printed on flexible surfaces such as plastics and clothes.

“We have developed unique characterization techniques such as atomic-force microscope potentiometry to explore fundamental mechanisms governing carrier transport and band structure of organic materials. The discovery of unexpected small fluctuations of the electronic band in the organic compound pentacene led us to initiate new research on so-called terahertz wave imaging sensors for applications that include security checks at airports,” says Nakamura.

Another area where flexible devices could

play an important role is in energy harvesting. A recent highlight is the design of flexible thermoelectric conversion materials and devices. Nakamura and colleagues have also discovered that high-purity thin films of fullerenes and some other materials exhibit a large thermopower (as defined by the Seebeck coefficient) of 100 mV/K, which is a hundred to a thousand times larger than normal values and could be used in high-efficiency thermoelectric devices to convert heat into electricity.

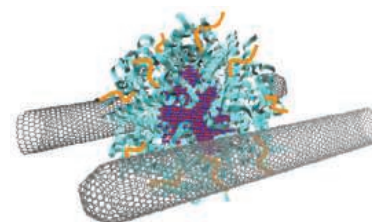
“Energy conversion and harvesting is



Masakazu Nakamura

important for powering flexible electronic devices on any surface,” explains Nakamura. “Recently we produced composite materials consisting of protein molecules bridging carbon nanotubes that could drastically improve thermoelectric conversion efficiency. The figure of merit, ZT —on which thermoelectric efficiency depends—is 2,200 times higher than conventional nanotubes.”

Nakamura Laboratory:
mswebs.naist.jp/LABs/greendevise/index_e.html



Fusion of Nanocarbon, Protein, and Semiconductors: A Novel Design of Thermoelectric Composites

Kumamoto University

Catalyzing the Next Generation of Multidisciplinary Researchers

Kumamoto University is located at the center of the island of Kyushu in southwestern Japan, approximately two hours by air from Tokyo. The university is one of Japan's most highly acclaimed higher education institutions, originating as one of the five institutions established during the Meiji era (in the nineteenth century) that united in 1949 to form the current university. It is renowned for its high academic standards and its research on AIDS, magnesium alloys, pulse power, and advanced life sciences. Its picturesque location is at the heart of Kumamoto City, famous for its rich culture, stunning mountain vistas, and the spring water from Mount Aso, an active volcano.

In October 2013, Kumamoto University was one of 22 institutes chosen by Japan's Ministry of Education, Culture, Sports, Science and Technology (MEXT) for the prestigious and highly competitive Program for Promoting the Enhancement of Research Universities.

"Of the 22 universities and institutes selected for this program, Kumamoto University is farthest from Tokyo," says Trustee and Vice President **Shinji Harada**, who is in charge of research and social cooperation. "The two major goals of the MEXT program at Kumamoto University are training university research administrators [URAs] and enhancing research at its internationally renowned institutes" (see sidebars, below).



Kumamoto Castle

Kumamoto University is adopting an integrated and holistic approach for the administration of the MEXT program. Specifically, the university has established the Priority Organization for Innovation and Excellence (POIE) as their unified management system. POIE is led by Kumamoto University President **Isao**

Taniguchi. "Ours is an 'all-university' initiative," explains Harada. "We have contributions from researchers in the life sciences, natural sciences, humanities, and social sciences. We expect the fruits of this program to benefit our undergraduates' education as well."

The history of research at Kumamoto University shows the truly open policy of securing a world-class research staff. "Approximately two-thirds of the faculty members at the university's medical school were recruited from other institutes in Japan," explains Harada. "This policy of head hunting is rare in Japan and is a trademark of Kumamoto University. We welcome active, highly motivated researchers from all over the world to join us as part of this program."

Kumamoto University:
www.kumamoto-u.ac.jp

Center for AIDS Research

The Center for AIDS Research (CAIDS) was established in 1997 as the first academic center in Japan devoted to studying HIV and acquired immunodeficiency syndrome (AIDS). With its broad and extensive expertise in the fields of immunology and drug development,



Masafumi Takiguchi

CAIDS has been contributing to the development of vaccines and new drugs, including the protease inhibitor drug, Darunavir. Recently, it served as the core for a

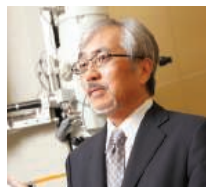
Global Centers of Excellence program, which promoted international research and provided world-class training opportunities. It focuses on immunology and strives for the development of new immune-based treatments, vaccines, and new drugs.

Director: Masafumi Takiguchi
www.caids.kumamoto-u.ac.jp/aidsnew/englishpage/

Magnesium Research Center

The Magnesium Research Center (MRC), established in December 2011, is focused on further enhancing the unique properties of magnesium (a light-weight metal) by developing magnesium alloys for applications such as aircraft construction. Highlights of MRC's recent research efforts include the realization of *KUMADAI* Heat Resistant Magnesium Alloy in 2003 and *KUMADAI* Non-flammable Magnesium Alloy in 2012.

Director: Yoshihito Kawamura
www.mrc.kumamoto-u.ac.jp (in Japanese)



Yoshihito Kawamura

Institute of Molecular Embryology and Genetics

Established in 1992, research at the Institute of Molecular Embryology and Genetics is focused on cutting-edge life and medical science from the point of view of developmental biology and human diseases. The institute comprises 30 primary faculty members in the three main divisions of Developmental Regulation, Stem Cell Research, and Organogenesis, as well as the Center for Organ Regeneration Research and Regenerative Medicine.

Director: Mitsuyoshi Nakao
www.imeg.kumamoto-u.ac.jp/en/index.html



Hidenori Akiyama

Institute of Pulsed Power Science

The Institute of Pulsed Power Science conducts interdisciplinary basic and applied research on pulsed power—the temporally controlled, instantaneous release of energy (electrostatic, magnetic, mechanical, or chemical). Research areas being pursued at the institute include medical and environmental applications using the biological effects of pulsed power (bioelectrics), and extreme material science using explosives and super-gravity facilities.

Director: Hidenori Akiyama
www.ipps.kumamoto-u.ac.jp/English/



Mitsuyoshi Nakao



Keo University is committed to the “protection of the global environment and the development of a sustainable, zero-waste society,” according to their 2012 Environmental Policy Statement, which informs the university’s research and teaching focus. Founded in 1858, it is one of the oldest and most prestigious private universities in Japan, offering courses in the arts, social sciences, physical sciences, engineering, and medicine. This large and influential academic institution has 10 undergraduate schools, 14 graduate schools, approximately 1,400 tenured faculty, and 33,000 students. The challenging research and education programs at the university reflect the spirit



Keio University Library (old building)



Shonan Fujisawa Campus

of “independence and self-respect” espoused by the university’s founder, **Yukichi Fukuzawa.**

Just two of these world-class programs are outlined below.

Keio University:
www.keio.ac.jp/index-en.html

Global Environmental System Leaders Program

“Our faculty, administration staff, and students are aware of their individual responsibility to contribute to a sustainable global ecosystem,” says **Yasushi Kiyoki** from the Graduate School of Media and Governance, and leader of the Global Environmental System Leaders (GESL) program. “Education and research at Keio University are intrinsically linked with environmental issues. The GESL program is an excellent example of our multidisciplinary approach to environment-related research.”

With full backing from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Program for Promoting the Enhancement of Research Universities, the GESL program aims to nurture global leaders with a deep understanding of social and technological issues, with the ultimate goal of spearheading global efforts to preserve the Earth’s ecosystem.

Students in the GESL program take a five year course—two years for their Master’s and three additional years for their Doctoral training—choosing major and minor specialties from four courses taught at the Graduate School of Media and Governance and Graduate School of Science and Technology. Notably, GESL students spend up to six months doing fieldwork at one of the partner institutes, which include Princeton University in the United States, the University of Cambridge in the United Kingdom, Tampere University of Technology in Finland, and the United Nations Environment Program (Asia Pacific Adaptation Network).

Kiyoki’s research on a Mathematical Model of Meaning (MMM) is central to the running of the GESL program. “MMM links the cyberworld with the physical world,” explains Kiyoki. “It enables context-dependent web searching for



Yasushi Kiyoki

environment-related media data, such as video and photographs.” GESL students share this highly

intercorrelated information with researchers at other institutes in real time, to analyze and monitor natural disasters. “We expect 30%–40% of the GESL graduates to join international institutes, such as the United Nations,” says Kiyoki. “This is Keio University’s contribution to environmental research and policy making.”

Global Environmental System Leaders Program:
gesl.sfc.keio.ac.jp

Challenges in Cross-Border Recycling in Asia

Eiji Hosoda is an economist who studies waste management, specifically the cross-border movement of recyclable products. “The mid-1980s saw major increases in exports of so-called end-of-life products from economically mature countries to nations such as China that had a strong demand for raw materials,” he explains.



Eiji Hosoda

The early, uncontrolled cross-border movement of recyclable products had two major effects in Asia. In Japan, strict

extended producer responsibility (EPR) laws were enacted in 1997 to recycle packages, home appliances, and end-of-life vehicles. Yet despite these laws, the outflow of recyclable goods to developing countries severely limited the reuse of valuable resources within Japan itself. This led to recycling businesses going bankrupt. The second issue was that end-of-life goods transported to other Asian countries were potential sources of pollution, such as “e-waste” from electrical goods.

“I realized that incinerators and other such technology alone would not be enough to prevent potential environmental problems,” says Hosoda. “We needed to develop an internationally viable system to deal with the whole process of cross-border recycling and waste management.”

In 2006, Hosoda and colleagues in Kitakyushu City, Japan, and Tianjin, China, joined in a three-year pilot project for recycling mixed plastics. The results were published in a 2009 report entitled, “A Guideline on Cross-Border Trading for Recycling: Kitakyushu-Tianjin Method.” The combination of using electronic tags for tracking the movement of plastics, establishing an institute to certify private companies, and the participation of representatives from the cities of Kitakyushu and Tianjin, have been key to the success of the project. “This model for waste recycling and management is globally transferable across national borders,” says Hosoda.

Hosoda Laboratory:
web.econ.keio.ac.jp/staff/hosoda/



Waseda University

Bold Initiatives of the Waseda Vision 150

With approximately 43,974 undergraduates, 9,357 graduate students, 4,362 international students, and 1,679 tenured teaching staff, Waseda University is one of Japan's largest research-based private academic institutes. The university is a member of the Research University 11, a consortium of 11 Japanese research-based universities established in 2009.

"The landmarks given in the Waseda Vision 150 define our road map until 2032, which is the 150th anniversary of our founding," says Vice President **Shuji Hashimoto**. "The funding from the Ministry of Education, Culture, Sports, Science and Technology [MEXT] Program for Promoting the Enhancement of Research Universities will be integrated into existing projects and infrastructure to realize the goals of our roadmap. The Waseda Vision 150 is our commitment as one of the leading universities in Asia to contribute to cutting-edge research and inspire education on a global scale."

The major targets of Waseda Vision 150 include increasing the number of international students from approximately 4,300 to 10,000, increasing the number of lectures given in foreign languages from approximately 7% to 50%, and increasing the number of tenured teaching posts to 2,000. The university is well situated for overseas researchers, with plenty of international schools and accommodations available in the area, and an English-speaking community that has already taken root.

As part of the MEXT program, Waseda University will hire eight university research administrators (URAs). The responsibilities of the newly appointed URAs will include planning and executing research projects based on objective analysis of international re-



Shuji Hashimoto (left) and Yoshiaki Fukazawa

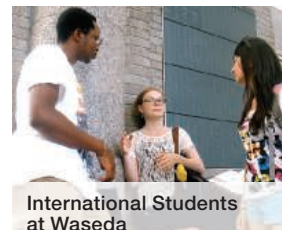
search trends, establishing research centers, supporting young researchers, distributing press releases, and dealing with the media. In addition to URAs, the Waseda University project will make use of the Waseda Research Institute (WRI) Corporation, a company set up by the university to comprehensively support research activities, supporting the promotion of cooperation with industry, academia, government, and the community by the university's research teams.

"All the new URA staff will be employed as members of faculty as part of the Center for Research Strategy," says **Yoshiaki Fukazawa**, senior executive director for the promotion of research and information technology. "URA career paths are still being established in Japan. We expect our URAs to be able to interface with researchers, administrators, and society. Their contracts are for five years initially, but we are considering the possibility of converting these positions into tenured posts."

Waseda University:

www.waseda.jp/top/index-e.html

www.waseda.jp/rps/kenkyu/index-e.html



International Students at Waseda

Researcher Spotlight

Hitoshi Kurumizaka, a faculty member at the Graduate School of Advanced Science and Engineering, joined Waseda University in 2003 following a postdoctoral fellowship at the National Institutes of Health (NIH) in the United States and a five-year stint as a research scientist at RIKEN in Saitama, Japan.

"I am currently studying how DNA is packaged into the tiny, microscopic space of the eukaryotic nucleus by histone proteins," says Kurumizaka. "More specifically, we want to clarify the crystal structure of specific histones associated with packing nucleosomes—the fundamental unit of DNA packing in eukaryotes. Histones hold the key to how we humans have a total of around 100 billion kilometers of DNA wound up in the nuclei of our cells."

To ensure specific functions, a precise chromatin architecture of DNA-protein complexes is required for centromeres—the attachment point for the chromosome to the mitotic spindle during cell division—to be generated on chromatin. A centromere-specific chromatin is produced

by the assembly of nucleosomes containing the centromere-specific histone H3 variant, CENP-A, a protein whose structure is not well understood.

In a 2011 *Nature* paper, Kurumizaka and colleagues successfully reconstituted the centromere-specific nucleosome with human CENP-A and determined its crystal structure. "In this ground-breaking study, we revealed the specific and common structural properties of the CENP-A nucleosome, compared with the canonical nucleosome," says Kurumizaka.



Hitoshi Kurumizaka

This is the first report describing the centromeric nucleosome structure at an atomic resolution. The results have important implications for understanding the molecular mechanisms underlying chromosome segregation, epigenetic inheritance of the chromatin domains, and functional chromatin formation—processes so important that their destruction can cause serious diseases, including cancer.

Kurumizaka Laboratory:

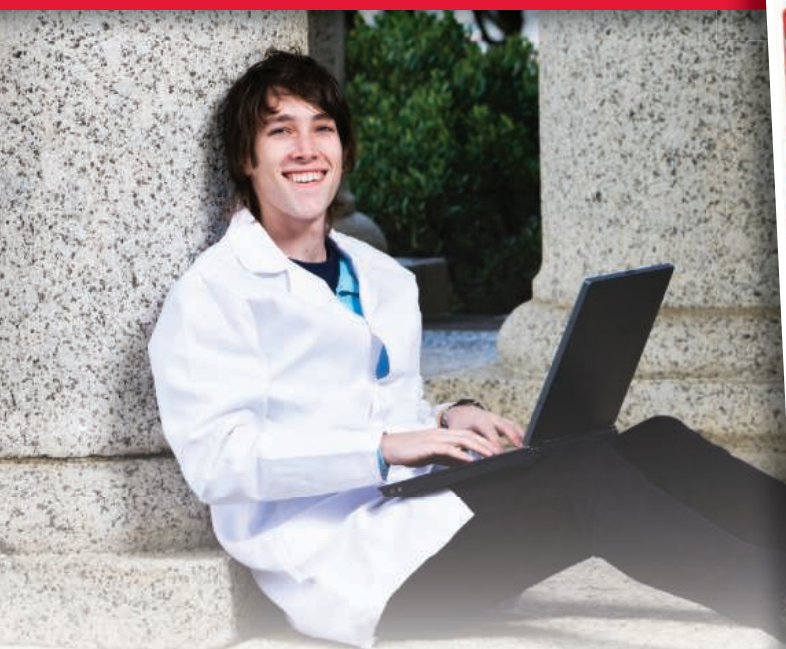
www.hrc.waseda.ac.jp/eng/member/kurumizaka/



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The Alexander von Humboldt Professorships are Germany's way of creating a beacon effect and energising its research landscape. Every year, the Alexander von Humboldt Foundation is offering ten of the world's leading researchers up to five million EUR each to create new or consolidate existing internationally visible research focus areas at German universities.

Academics of all disciplines are eligible for an Alexander von Humboldt Professorship, provided that they are established abroad and recognised internationally as top-class researchers. They will be nominated by German universities – where appropriate in cooperation with non-university research institutions. Each Alexander von Humboldt Professorship will be sponsored for a period of five years on the premise that the university presents a convincing strategy to sustain the position once the funding period has come to an end.

This will allow new, long-term research groups to be established, conducting cutting-edge international research. The German Ministry of Education and Research is supporting this programme.

The Humboldt Foundation actively promotes equal opportunities and therefore particularly welcomes nominations on behalf of leading female academics.

More information: www.humboldt-foundation.de/ahp-en.

Closing dates for nominations: 15 April and 15 October.

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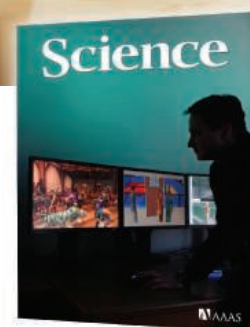


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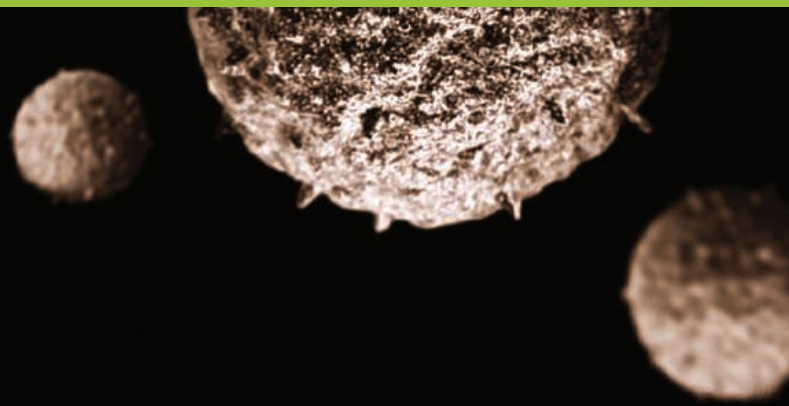
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A New Breed of Cancer Researcher

As our understanding of cancer has gone from simplistic to complex, so too has research on treatment and detection. In a field once dominated by scientists with biomedical backgrounds, cancer research today includes engineers, chemists, and physicists. To land a job in any emerging area of cancer research, interdisciplinary training is becoming increasingly important. Luckily for job seekers, educational institutions are rising to meet the need. **By Gunjan Sinha**



When **Martin Pule** landed a Fulbright scholarship 14 years ago to study at the Center for Cell and Gene Therapy in Baylor College of Medicine, he never anticipated that he would be working in one of the hottest fields of cancer research today: immunotherapy. At Baylor, Pule began training in Malcolm Brenner's laboratory—one of the pioneers of engineered T-cell treatments. In 2008, Brenner's team published one of the first studies demonstrating that T cells could be taken from a patient with neuroblastoma, engineered to express tumor-specific receptors, and then administered back to the patient as a treatment. At the time most cancer researchers considered T-cell therapy a “fantastical and unrealistic thing to do,” Pule recalls. It was “cumbersome and seemed a lot less feasible than treating patients with cancer-fighting antibodies.” Fast-forward to 2014 and immunotherapy is one of the most promising, novel ways to treat cancer to emerge in over a decade.

Pule's career path is typical of many scientists working at the crossroads of immunology, gene therapy, and cancer. Trained in medicine, his research focus came from a coalescing of chance decisions with the march of scientific progress. While Pule largely acquired his varied expertise along the way, interdisciplinary training is the catch phrase for scientists entering emerging fields of cancer research today.

“There's a strong perceived need among cancer research funding bodies that new modes of education and skill sets are necessary,” says **Bennett Goldberg**, director of Boston University's (BU) Center for Nanoscience and Nanobiotechnology, an interdisciplinary center that brings together academic and industrial scientists and engineers to develop nanotechnology applications in biomedicine. “There's been a huge push over the past five years to apply more physical science and engineering to biomedicine in general.”

BU's center was jump started by a \$2 million grant from the National Cancer Institute (NCI) Alliance for Nanotechnology in Cancer in 2010 and is one of six cancer nanotechnology training programs supported by NCI. NCI has spearheaded several additional initiatives to promote research on nanotechnology applications in cancer, which include funding centers of nanotechnology excellence and cancer nanotechnology platform partnerships.

NCI isn't alone in promoting cross-disciplinary approaches to developing new cancer treatments. Memorial Sloan Kettering Cancer Center (MSKCC) in New York City has set up several collaborative research centers focused on promoting research across scientific disciplines; the Massachusetts Institute of

Technology's (MIT's) David H. Koch Institute for Integrative Cancer Research in Cambridge, Massachusetts has been fully operational since 2010; and other institutions, such as the Fred Hutchinson Cancer Research Center in Seattle, Washington, offer dual-mentor fellowships in cancer research. While the approach to interdisciplinary training or research may differ by institution, the goal is the same: to encourage and facilitate collaboration across different areas of science to develop novel approaches for cancer detection and treatment. Some of the most fervent activity is in the fields of immunotherapy, nanotechnology, and epigenetics.

IMMUNOTHERAPY

Scientists have tried for decades to coax the body's immune system to stamp out cancer cells but have had little clinical success. After years of setbacks, promising results finally began flowing out of clinical trials a few years ago with a therapy that uses chimeric antigen receptors (CAR)—receptors engineered to recognize specific targets on cancer cells.

CARs typically contain a portion of a monoclonal antibody, which recognizes a specific tumor antigen, and are linked to signaling molecules inside the T cell. To use these receptors as a therapy, T cells must first be isolated from a patient's blood and engineered ex vivo using gene-therapy vectors. Once returned to the patient, the T cells recognize and bind to target antigens on cancer cells, stimulating the signaling molecules that in turn instruct the T cell to activate, divide, and attack cancer cells.

Recently, stunning clinical trial results showing that CAR therapy could help dramatically shrink tumors in patients who had failed to respond to conventional treatments led to cancer immunotherapy being dubbed *Science's* “Breakthrough of the Year” in December 2013 (news.sciencemag.org/breakthrough-of-the-year-2013).

The excitement isn't restricted to the scientific community; industry **continued>**

“There's been a huge push over the past five years to apply more physical science and engineering to biomedicine in general.”

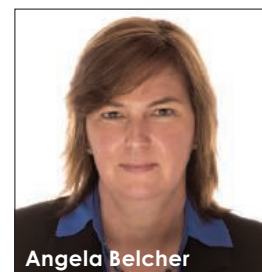
—Bennet Goldberg

Upcoming Features

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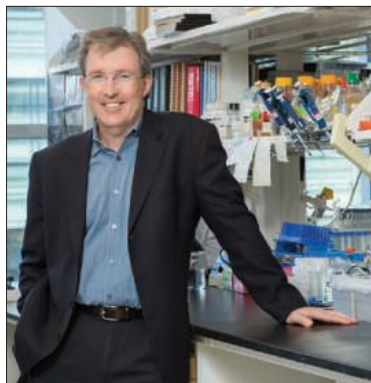
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Angela Belcher

Any type of interdisciplinary training is “a good way to position yourself for something that is growing in importance.”
—Tyler Jacks



is also on board. In December, The Fred Hutchinson Cancer Research Center, MSKCC, along with pediatric partner Seattle Children’s Research Institute teamed up to form Juno Therapeutics, a Seattle-based biotechnology company aiming to develop novel cancer immunotherapies. Juno was launched with an initial investment of \$120 million—one of the largest pots of seed funding for a biotech startup in history. Juno’s incarnation followed Novartis’ 2012 exclusive global collaboration agreement with the University of Pennsylvania to research, develop, and commercialize targeted CAR immunotherapies to treat different types of cancer.

“T-cell engineering has gone from being something of a cottage industry with a small group of investigators tinkering along to a field that has completely exploded,” says Pule.

After Baylor, Pule returned to Europe to finish his clinical training and to set up his own lab at University College London (UCL), where he is now a clinician scientist in the UCL Cancer Institute’s Department of Hematology. Pule’s lab is coordinating the Advanced T-cell Engineered for Cancer Therapy (ATECT) consortium—a €6 million (\$8.13 million) five-year research collaboration funded by the European Commission that is aimed at improving CAR therapy.

Most T-cell engineering approaches are designed to simply introduce genes into cells using viral vectors, Pule explains. But ATECT scientists have combined viral vector technology with a proprietary genome-engineering tool developed by Paris, France-based Collectis, an ATECT partner, called transcription activator-like effector nuclease (TALENs), enabling them to also disrupt or delete genes. Using this gene editing technology, researchers have the potential to disrupt the genes that cause a patient’s body to attack or reject donated T cells.

Currently, to avoid immune system rejection, CART cells are made for each patient individually using their own cells. An “off the shelf” T-cell cancer therapy, however, would eliminate the need to personalize every treatment. It would also enable CAR therapy to be mass-produced and would greatly facilitate access to what remains a highly complex experimental therapy. Collectis is currently developing several off-the-shelf CAR T-cell products.

For job seekers, the immunotherapy’s success means opportunities. “The easiest way to get into this field now would be to land a position in one of centers that are doing this kind of work,” says Pule. “A wide range of people are needed.” Among them are molecular biologists and protein engineers who make the receptors, immunologists who do the in vitro and preclinical research, virologists who make the viral vectors, and skilled specialists who can manufacture vectors and cells according to Good Manufacturing Practice guidelines. That said, “a lot of know-how and skills are just learned on the job,” he adds.

Seth A. Ettenberg, head of biologics for oncology at Novartis Institutes for Biomedical Research in Cambridge, Massachusetts, agrees. Ettenberg is in the process of assembling a team of scientists to interface with researchers at the University of Pennsylvania with whom the company is co-developing CAR immunotherapies. “The main question that we ask job applicants is: ‘Do you have a story to tell?’” In hiring early career scientists, Novartis looks for candidates who

have demonstrated that they ask big picture questions and do rigorous hypothesis driven research, he says. Ideally, job candidates will also have published in a top journal, he adds, although this criterion generally extends across all areas of the company. A more unique skill required for the cancer immunotherapy group is the ability for intense teamwork. Because cell-based therapies are still experimental, Novartis has immunologists and cell manufacturing specialists working side-by-side. This differs from their development process for small molecules and antibody-based drugs, where manufacturing practices are well understood, says Ettenberg. Therefore, a candidate’s communication skills are of the utmost importance.

Novartis’ hiring managers typically speak to candidates several times over the phone before inviting them to give a presentation. The presentation can make or break a person’s prospects, Ettenberg adds. “We really look at how well thought out the presentation is and how they handle questions. Are they dismissive, aloof, do they say ‘I don’t know’ when they really don’t know?” If it goes well, candidates are then invited for a full day of interviews. For senior scientists, interdisciplinary training is important for the tasks they will be required to do. For creative and excited scientists just starting out, however, there are many skills one can learn on the job, Ettenberg says.

NANOTECHNOLOGY

Engineered T cells aren’t the only emerging technology promising to transform cancer treatment. Nanotechnology—the science of manipulating matter at the nanoscale to create devices with novel chemical, physical, and biological properties—also has the potential to radically change how cancer is diagnosed and treated. From new imaging agents to new modes of drug delivery, “cancer nanomedicine” is another exploding field of cancer research.

NCI recognized the potential of nanotechnology to improve cancer treatment and detection as early as 2004 when it formed the NCI Alliance for Nanotechnology in Cancer. In addition to several other nanotechnology projects, NCI Alliance currently supports nine centers of cancer nanotechnology excellence, six cancer nanotechnology training programs, and 12 cancer nanotechnology platform research partnerships aimed at addressing major barriers and fundamental questions in cancer.

At the Koch Institute for Integrative Cancer Research, a recipient of NCI Alliance grant money, **Angela Belcher**, works on improving nano-based imaging of cancerous tissue. Current practices employ computed tomography, magnetic resonance imaging, or ultrasound to image soft tissues. Belcher’s lab is trying to develop a less expensive method that will enable smaller tumors to be imaged at greater tissue depths than is currently possible with these technologies. Her approach uses genetically modified bacteriophages that bind specific cancer cell receptors and that carry a single carbon nanotube which fluoresces when exposed to near-infrared light. Her team has already demonstrated that the technique enables imaging of tissues as deep as 3 cm in animals, but should be able to image up to 10 cm, she says.

“Angie is a very good example of the type of people we like to have working here,” says **Tyler Jacks**, director of the Koch Institute. “She has a deep background in materials science but is working at the convergence of life science and engineering.”

Belcher, who has a joint appointment at the MIT Department of Materials Science and Biological Engineering, is a materials chemist by training. She accepted the joint appointment two and a half years ago after she collaborated on a cancer-related project with MIT biomedical engineer Bob Langer. At the Koch Institute, her task is to use engineering to solve challenges facing cancer researchers. The interdisciplinary environment at Koch is a good fit for her, says Belcher, since she has had interdisciplinary training from early on—she designed her own major as an undergraduate at the University of California, Santa Barbara. “I’ve always asked the question: What different disciplines are helpful for addressing the problem you want to solve?” she explains. **continued>**



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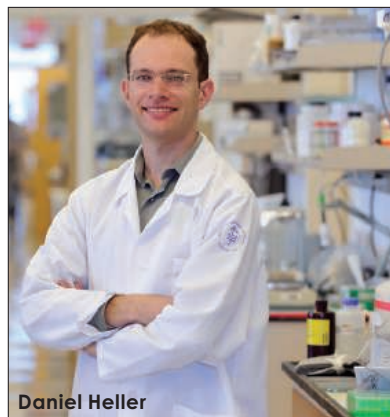
David H. Koch Institute, Massachusetts Institute of Technology
ki.mit.edu

Novartis Institutes for BioMedical Research
www.nibr.com

Memorial Sloan Kettering Cancer Center www.mskcc.org/research/ski

The Gurdon Institute, University of Cambridge
www.gurdon.cam.ac.uk

University College London
www.ucl.ac.uk



Daniel Heller

Daniel Heller, on the other hand, came to cancer nanomedicine with a background almost exclusively steeped in physical science. Heller came to the Molecular Pharmacology and Chemistry Program at MSKCC after finishing his Ph.D. in chemistry at the University of Illinois at Urbana-Champaign. Except for a brief stint as a postdoc in Bob Langer's lab at MIT, he had never worked in biomedicine.

Heller landed at MSKCC almost by accident. While at MIT, he met his future MSKCC head, who suggested that he apply for a job, at a meeting. "I had no idea that Sloan Kettering was even an option," recalls Heller, who had been applying exclusively to universities. In retrospect, "not only is it the best place for me, but it is probably the best place for a lot of people who don't even know it," he says.

The reason: need. In most engineering departments "there are a lot of people making hammers, but there aren't a lot of nails nearby," explains Heller. "Now, I'm in a place where there are a lot of nails, and I'm one of a few hammers. That's an exceptional position to be in."

At MSKCC, Heller focuses on developing nanoscale molecular sensors and targeted therapeutics. For example, his team is currently making optical sensors from carbon nanotubes in order to quantify metabolite levels in living cells—a tool that has no existing counterpart. Indeed most of the projects in his lab involve creating tools that enable scientists to study what had not been previously possible because the technology was nonexistent. In coming up with project ideas, Heller has found that the ability to have on-going dialogues with cancer clinicians who also work on site makes it easier for him to understand the problems he is tasked with solving.

Heller's lab consists of a hodge-podge of expertise, including physicists, chemists, and biologists. This type of interdisciplinary team often poses language barriers at first, but these can break down over time through working together. Teamwork is key, he says. "You can't be too introverted." For scientists working in any discipline and looking for a job in cancer research, there are opportunities in areas and places that are not obvious. "Talk to people and look at alternative places to work that are far outside of your field," advises Heller.

EPIGENETICS

Interventions that alter the epigenome of cancer cells present yet another novel cancer treatment approach. A cell's epigenome is a secondary level of genetic modification that does not affect the integrity of genes, but impacts when and where they will be expressed. As with the mutations that change the genetic code, epigenetic abnormalities can also drive cancer. However, unlike genetic mutations, epigenetic changes are reversible, and as such, potentially correctable with therapeutics. There are already four U.S. Food and Drug Administration (FDA)-approved drugs that alter the epigenetic profile of cells to treat cancer and at least 30 more investigational drugs in on-going cancer clinical trials, according to current data available on clinicaltrials.gov.

inflammation was actually active against leukemia cells, specifically mixed-lineage leukemia, the most common form of leukemia in babies. The drug mimics an epigenetic tag in bone marrow cells that prevents leukemia-causing genes from being activated and is now in clinical trials. "We helped [GSK] understand the drug's mechanism of action," says Kouzarides, "when the company didn't have the expertise in this area."

Kouzarides is a biologist by training, having earned a Ph.D. studying viruses in the Department of Pathology at the University of Cambridge. Over the years, however, he became specialized in cancer genetics. Today his laboratory focuses on the epigenetics of histone proteins, which package DNA, and how epigenetic mechanisms regulate gene expression in cancer cells. The work done in his group today requires a very broad range of skills, he says. For example, if one of his researchers identifies an important cancer-related signaling pathway, they would benefit from a knowledge of chemistry so they can communicate their needs to companies that have libraries of molecules that might target their pathway of interest, explains Kouzarides. They might also have to interact with cancer clinicians as well as generate models of the disease to better understand the pathway.

Kouzarides isn't suggesting that people interested in working in the field of cancer epigenetics become a "jack of all trades." In fact, quite the opposite: "everyone has a type of science that they do, and no one can do everything," he explains. "Certainly having one or two major skills is important, but the rest you can pick up from collaborations."

BU's Goldberg concurs: "You have to have depth in one area to develop scientific acumen," he says, "since there's a confidence that comes with depth in one field." Only then can one go on to develop breadth across several areas, he explains, which is something nanotechnology training programs help to provide.

While epigenetics, cancer nanomedicine, and immunotherapy are important new directions for cancer research, they certainly are not the only ones. The Koch Institute, for example, received NCI funds to establish interdisciplinary training programs in cancer systems biology and a physical sciences oncology center in which network theory and mathematical and computational modeling are being brought to bear on cancer research. To work at the crossroads of any of these areas, "having a very broad perspective is becoming increasingly attractive," says Jacks.

For Ph.D. graduates in any of the sciences who are interested in cancer research, there are many ways to gain that broad perspective including interdisciplinary fellowships that require students to choose mentors from different disciplines, postdoctoral training programs outside of one's field, and institutional training programs. According to Jacks, any type of interdisciplinary training is "a good way to position yourself for something that is growing in importance."

Gunjan Sinha is a freelance writer living in Berlin, Germany.

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Applicants should provide electronic copies (only) of a cover letter stating area of expertise and qualifications, synopsis of professional goals, research interests, curriculum vitae, and e-mail addresses for three references to CancerResearch@ouhsc.edu.

Confidential enquiries may be addressed to **Danny N. Dhanasekaran, PhD, Deputy Director for Basic Research**, at danny-dhanasekaran@ouhsc.edu.

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Please send curriculum vitae, a list of three or more references, and a cover letter outlining your research interests electronically to: **Stanton L. Gerson, MD, Director, Case Comprehensive Cancer Center, cancersearch@case.edu**. Please include "Cancer Research Faculty Search" in the subject line.

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Successful candidates will be expected to interact closely with ongoing basic and translational research, particularly research using human tissue. Candidates with interests in Gastrointestinal Cancers (colon cancer, esophageal cancer, pancreas cancer) that could align with the Case GI SPORE are particularly encouraged, as are candidates with interests in glioma, breast cancer, ovarian cancer, or hematologic malignancies would align with cancer center scientific programs. Physician scientists are particularly encouraged to apply. Primary appointment will be in the department of training, the cancer center or the department of Genetics.

Please send curriculum vitae, a list of three or more references, and a cover letter outlining your research interests, electronically to: **Stanton L. Gerson, MD, Director, Case Comprehensive Cancer Center, cancersearch@case.edu**. Please include "Cancer Genetics" in the subject line.

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The Department of Systems Biology at The University of Texas MD Anderson Cancer Center invites applicants for a term tenured or term tenure-track, full-time academic appointment at the rank of assistant professor or higher. The position offers the opportunity for professional growth and development

by joining an exciting and active group of basic scientists and translational investigators. The successful candidate will closely interact with the many ongoing research programs within the Department of Systems Biology and is expected to maintain an active funded research program in Systems Biology. MD Anderson is ranked as the #1 cancer center in the U.S. and is known for excellence in collaborative research and cross-disciplinary training.

This research faculty position will advance the program of Cancer Systems Biology through cutting-edge methods including, but not restricted to, single cell biology, synthetic biology, genome-scale biology (network biology, omics and in particular metabolomics) or predictors of prognosis and response to therapy. The research faculty position is expected to participate in and collaborate with active program(s) ongoing in the department in this discipline. Specifically, the faculty member will be responsible for the day-to-day running of their own research laboratory and for planning and executing experiments. In addition, the individual will be required to procure and maintain independent peer review research funding, design and analyze laboratory research projects, supervise assigned laboratory personnel, teach fellows/students, and initiate, as well as help, write grants and papers.

Start-up funds and excellent laboratory space are available. The successful candidate will benefit from closely interacting with the many ongoing basic and translational research programs of the Department of Systems Biology. In addition, the candidate will be expected to develop an original, creative and independent research program and have the opportunity to participate in ongoing research with world-class clinical and laboratory researchers.

Applicants should hold a Ph.D., M.D. or M.D./Ph.D., and at least 5-10 years of related experience and/or training; or equivalent combination of education and experience. Must have several years of experience working in a research environment where duties involve scientific research disciplines, at a UT component or at another academic institution, or have applicable experience deemed equivalent. Must show evidence of consistent research of high quality, a dedication to a career in cancer research, and a proven track record of original research and publications. In addition, applicants must demonstrate potential to obtain extramural funding.

MD Anderson offers a highly attractive recruitment package and the unrivalled scientific environment of the Texas Medical Center. Please forward curriculum vitae, statement of research interest and a list of three to five references to:

Gordon Mills, M.D., Ph.D., Chair
The University of Texas MD Anderson Cancer Center
Department of Systems Biology, Unit 950
South Campus Research Building II
P. O. Box 301429
Houston, TX 77230-1429
E-mail: gmills@mdanderson.org

Applications will be accepted until position is filled.

MD Anderson is an equal opportunity employer and does not discriminate on the basis of race, color, national origin, gender, sexual orientation, age, religion, disability or veteran status except where such distinction is required by law. All positions at The University of Texas MD Anderson Cancer Center are security sensitive and subject to examination of criminal history record information. Smoke-free and drug-free environment.



Faculty Positions in Viral Immunopathogenesis at the Vaccine & Gene Therapy Institute of Florida

The Vaccine & Gene Therapy Institute of Florida (VGTI Florida) is seeking to recruit outstanding immunologists/virologists to direct research programs in basic and translational human immunology. Priority areas of research include immunopathogenesis, emerging viral pathogens including influenza and flaviviruses, vaccine development, adjuvants, and inflammation. We are seeking scientific leaders in the areas of molecular virology, immune response to infection and antiviral/vaccine strategies. Priority will be given initially to established investigators with vigorous research programs investigating HIV, influenza, Dengue and emerging viruses. VGTI Florida is one of the internationally recognized research institutes invited to locate to Florida as part of a State-sponsored initiative to enhance biomedical research. Research at VGTI Florida focuses on human innate and adaptive immune responses to infectious diseases and cancer.

Research themes at VGTI Florida include:

- HIV-1 and emerging viral pathogens
- Vaccine development and adjuvants
- Cancer Immunology and immunotherapy
- Inflammation and diseases of aging

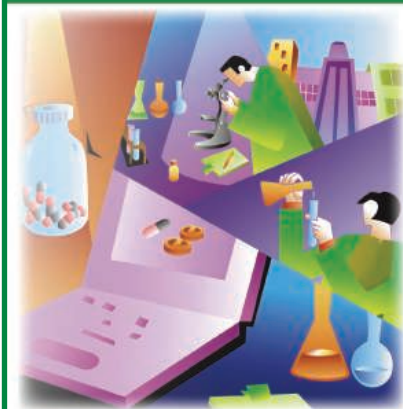
VGTI Florida occupies a new 100,000 sq. ft. state-of-the-art facility in Port St. Lucie, FL, located on the sunny Atlantic coast in a Biotech corridor just north of Palm Beach. Successful candidates (PhD and/or MD) will have an established extramurally-funded research program and a strong publication record in one of the priority areas described above. The positions have highly competitive salary and startup packages, with access to cutting edge Genomics, Bioinformatics and Flow Cytometry core facilities as well as BSL3/ABSL3 containment facilities within the Institute. For more information, including a description of the Faculty and their research interests, please visit: www.vgtifl.org. Qualified candidates should submit their curriculum vitae, a 2-page description of their proposed research program and the names/contact information of three references to:

Jill Hackett
Executive Director, Human Resources
Vaccine & Gene Therapy Institute of Florida

Applications must be sent via email to: search@vgtifl.org. Review of applications will commence immediately and continue until the positions are filled.

VGTI Florida is an Equal Opportunity Institution committed to recruiting, hiring, and promoting qualified minorities, women, individuals with disabilities, and veterans.

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Edith Sanford™
BREAST CANCER

Director Edith Sanford Breast Cancer Research

We seek an established clinical oncology investigator with a focus on clinical and translational breast cancer research to direct the Edith Sanford Breast Cancer Research program. The successful candidate will have the MD or MD/PhD degrees and will demonstrate a record of leadership and collaboration as part of a trans-disciplinary team. The Edith Sanford Breast Cancer initiative is the home of a \$100 million gift from our generous benefactor, Mr. Denny Sanford. The Director of Edith Sanford Breast Cancer Research will oversee development and implementation of the clinical and translational research program of the Edith Sanford Breast Cancer initiative. This initiative focuses heavily on the application of genomic medicine/forward genomics in pursuit of the goal of personalized medicine for breast cancer patients within the Sanford Health system and ultimately for patients across the nation. As the Director of Edith Sanford Breast Cancer Research, the successful physician-scientist leader will help drive the transformation of the Edith Sanford Breast Cancer workforce toward a clinical research-focused, genomic medicine-centered care delivery leader regionally and nationally.

Please send a letter of interest, curriculum vitae and a list of three references to:

H. Eugene Hoyme, MD
Chief Academic Officer and President of Research
Sanford Health, Route Number 5031
PO Box 5039
Sioux Falls, SD 57117-5039

AA/EOE

Keck School of Medicine of USC

Tenure-track Associate and/or Full Professors at the

University of Southern California
Norris Comprehensive Cancer Center and
Department of Biochemistry and Molecular Biology

The University of Southern California is seeking one or more outstanding cancer research scientists to fill new tenure-track faculty positions at the rank of Associate or Full Professor in the Department of Biochemistry and Molecular Biology at the USC Norris Comprehensive Cancer Center and Keck School of Medicine. Successful candidates should have well-established research programs and be considered leaders in their fields. They will be housed in the USC Norris Comprehensive Cancer Center, one of the longest-standing NCI-designated Comprehensive Cancer Centers, and will perform basic and/or translational cancer-related research that falls within the broad scope of topics covered by the Department of Biochemistry and Molecular Biology. This would include, but is not strictly limited to: biochemistry and molecular biology; genetics and genomics; epigenetics and epigenomics; gene regulation and signal transduction; and cancer-oriented developmental and stem cell biology. Faculty appointments will be made at the Full Professor or Associate Professor ranks commensurate with experience. We especially encourage applicants with a track record of running a funded, independent laboratory.

To apply, please visit our web site <http://jobs.usc.edu/postings/18085> and submit a single PDF file containing the following information by **June 1, 2014**: a cover letter, Curriculum Vitae, 1-page statement of future research plans, and names and contact information of 3 references.

USC values diversity and is committed to equal opportunity in employment. Women and men, and members of all racial and ethnic groups are encouraged to apply.

**Chair in Inflammation
Princess Margaret Cancer Centre**

Princess Margaret Cancer Centre and the Arthritis and Autoimmunity Research Centre (AARC) at the University Health Network in Toronto, invite highly productive individuals to apply for the position of Scotiabank Chair in Inflammation Research at either the Scientist or Senior Scientist level.

Research interests focused on inflammation pertaining to tumor biology and/or autoimmunity are encouraged. Applicants must have an M.D. and/or Ph.D. degree(s) and a proven track record, as evidenced by a number of high impact publications. The successful candidate will be expected to establish an original, competitive and independently funded research program, and to have a commitment to education in immunology.

Princess Margaret Cancer Centre is the largest centre for cancer research in Canada covering the full spectrum of applied and fundamental research. The AARC is the most comprehensive research centre for autoimmune diseases in Canada, combining clinical and applied studies with basic science research. The downtown location provides an extraordinarily rich scientific environment, adjacent to other major academic institutions including the University of Toronto St. George campus, the Toronto General Research Institute, the Hospital for Sick Children, the Samuel Lunenfeld Research Institute, the Toronto Western Research Institute, and the Ontario Institute for Cancer Research,

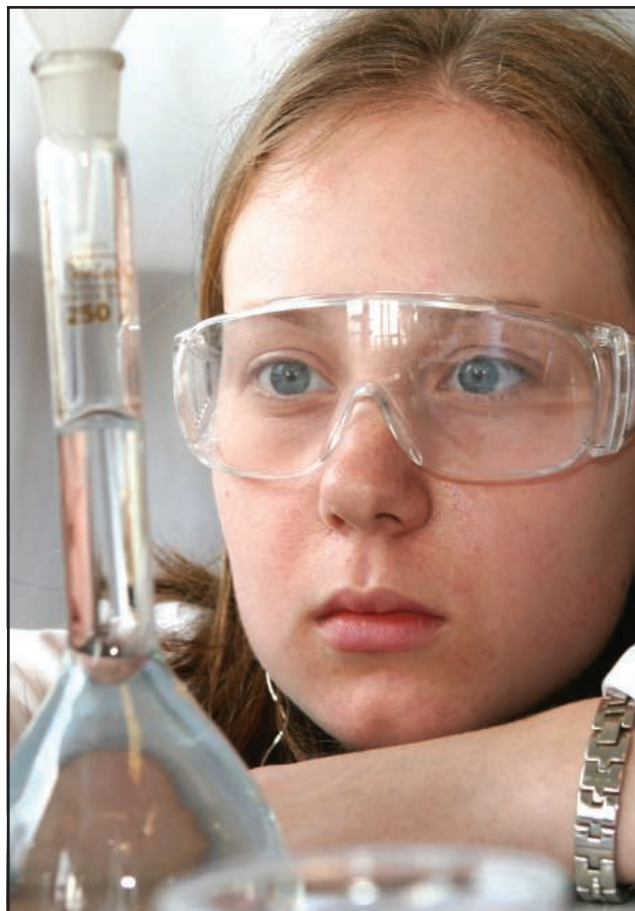
The successful applicant will receive full salary commensurate with the position and a generous start up package. Applicants will also be eligible for appointment at the Associate or Full Professor level in the Department of Immunology at the University of Toronto.

Interested candidates should send their CV and a 2 page description of research interests to:

**Inflammation Chair Search Committee,
Princess Margaret Cancer Centre
620 University Ave
Toronto ON M5G 2M9
cwells@uhnresearch.ca**

This search will remain open until a suitable candidate is identified.

The Princess Margaret Cancer Centre is the Research Institute of Princess Margaret Hospital, which, along with the AARC, Toronto General Hospital, Toronto Western Hospital and Toronto Rehabilitation Institute are members of the University Health Network, an Equal Opportunity Employer.



**AAAS is here –
promoting universal science literacy.**

In 1985, AAAS founded Project 2061 with the goal of helping all Americans become literate in science, mathematics, and technology. With its landmark publications *Science for All Americans* and *Benchmarks for Science Literacy*, Project 2061 set out recommendations for what all students should know and be able to do in science, mathematics, and technology by the time they graduate from high school. Today, many of the state standards in the United States have drawn their content from Project 2061.

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To learn more, visit
aaas.org/plusyou/project2061



There's only one

DR. SHIRLEY MALCOM



To Dr. Shirley Malcom, born and raised in the segregated South more than 65 years ago, a career based on her studies in science seemed even less likely than the launch of the Soviet's Sputnik. But with Sputnik's success, the Space Race officially started and, in an instant, brought a laser-like focus to science education and ways to deliver a proper response. Not long after, Dr. Malcom entered the picture.

Although black schools at the time received fewer dollars per student and did not have sufficient resources to maintain their labs at a level equivalent to the white schools, Dr. Malcom found her way to the University of Washington where she succeeded in obtaining a B.S. in spite of the difficulties of being an African American woman in the field of science. From there she went on to earn a Ph.D. in ecology from Penn State and held a faculty position at the University of North Carolina, Wilmington.

Dr. Malcom has served at the AAAS in multiple capacities, and is presently Head of the Directorate for Education and Human Resources Programs. Nominated by President Clinton to the National Science Board, she also held a position on his Committee of Advisors on Science and Technology. She is currently a member of the Caltech Board of Trustees, a Regent of Morgan State University, and co-chair of the Gender Advisory Board of the UN Commission on Science and Technology for Development. She has held numerous other positions of distinction and is the principal author of *The Double Bind: The Price of Being a Minority Woman in Science*.

Of her active career in science, Dr. Malcom says, "I guess I have become a poster child for taking one's science background and using that in many other ways: we ask questions; we try to understand what we find; we consider what evidence we would need to confirm or refute hypotheses. And that happens in whatever setting one finds oneself."

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The University of Texas Southwestern Medical Center is seeking outstanding candidates interested in becoming the next Chair of the Department of Physiology. Applicants should have an exceptional record of research achievement in areas relevant to modern physiology, as well as broad vision and strong leadership skills. Resources commensurate with these qualifications will be made available to the successful candidate. The Department occupies new laboratory space and has existing strengths in multiple areas of physiological research.

Qualified candidates are encouraged to submit a curriculum vitae to the chair of the search committee, **Dr. Eric N. Olson**, via email at: eric.olson@utsouthwestern.edu

UT Southwestern Medical Center is an Affirmative Action/Equal Opportunity Employer. Women, minorities, veterans and individuals with disabilities are encouraged to apply.



The Aaron Diamond AIDS Research Center
Affiliate of The Rockefeller University

The Aaron Diamond AIDS Research Center (ADARC), the world's largest private HIV/AIDS medical research facility, is seeking candidates for the position of Chief Executive Officer and Scientific Director.

The successful candidate must be of high scientific caliber in the field of HIV research and have led a successful independent research program in an academic or industry-related environment. In addition, s/he must have the standing to be appointed to the position of Head of Lab at Rockefeller University. The candidate must also have experience or demonstrated ability to manage substantial resources, scientific and administrative staff and in leading fundraising efforts.

The major responsibilities of the Chief Executive Officer and Scientific Director will be to work with:

- The Board of Directors and its Committees in the overall administration of ADARC. S/He will assure corporate governance, develop policies, create short and long-term plans and assume the lead for implementation and follow-up.
- The External Scientific Advisory Board (SAB) to evaluate the scientific progress of ADARC's Staff Investigators and strategize on the future scientific direction and growth of the organization.
- ADARC Faculty to provide guidance on individual scientific programs, help to secure program stability and funding.
- Senior Management, by delegating responsibilities appropriately to guarantee the accountability of ADARC to its diverse constituents and to oversee administrative and financial activities to ensure a smooth functioning, efficient and financially secure organization. S/He will have final executive decision-making authority for operation of the Center.

Application: Those interested should initiate the application process by sending a cover letter detailing previous experience and reasons for interest in the position, a curriculum vitae, and the names and contact information of 5 professional references. Direct all inquiries and applications to HR@adarc.org with the identifier 'Chief Executive Officer/Scientific Director' in the subject line.

ADARC is an Equal Opportunity Employer and will not discriminate against any employee or applicant on the basis of age, color, disability, gender, national origin, race, religion, sexual orientation, veteran status, or any classification protected by federal, state, or local law. Consistent with its obligations under federal law, each company that is a federal contractor or subcontractor is committed to taking affirmative action to employ and advance in employment qualified women, minorities, disabled individuals, special disabled veterans, veterans of the Vietnam era, and other eligible veterans.

ADARC is a smoke-free and drug-free environment.

Science Advances AAAS

NEW POSITION Editor, *Science Advances*

The American Association for the Advancement of Science (AAAS), publisher of the *Science* family of journals, seeks a well-respected scientist with an entrepreneurial spirit, broad research interests and networks, impeccable judgment in scientific excellence, and a passion for scientific communication to become the inaugural Editor for our new digital, open-access journal, *Science Advances*, spanning the fields of science, technology, engineering, mathematics, and the social sciences.

The new Editor ideally will have a PhD in a relevant scientific discipline, ten or more years of research experience, and some prior involvement with scientific journal editing. Reporting to the Editor-in-Chief of *Science*, the Editor will be responsible for providing overall editorial direction and strategy for the new journal, defining its scope, and serving as its champion. S/he will lead a team of Associate Editors based at academic and research institutions and establish the highest quality of the peer-review process, ensure timely decisions, and build a reputation for the new journal as a preferred choice for disseminating important new discoveries. The position is full time, although it could potentially be shared by two part-time editors.

Please visit our job information website <http://www.aaas.org/page/employment-aaas> to get more information and to apply online.

AAAS is an Equal Opportunity Employer.

PRIZE



The 2014 (30th) International Prize for Biology



Calling for Nominations

This year's research field: Systematic Biology and Taxonomy

Please access at: <http://www.jsps.go.jp/english/e-biol>

Deadline: May 16, 2014

- The International Prize for Biology was established in 1985 to commemorate the sixty-year reign of Emperor Showa and his longtime devotion to biological research.
- The Prize is awarded each year to an individual who has made an outstanding contribution to the advancement of basic research in a field of biology.
- The Prize consists of a medal and an award of 10-million yen (US\$100,000 at 100 Yen/dollar).

Recent Years Prize Winners



2013
Dr. Joseph Felsenstein
(Biology of Evolution)



2012
Dr. Joseph Altman
(Neurobiology)



2011
Dr. Eric H. Davidson
(Developmental Biology)



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Every month, over 400,000 students and scientists visit ScienceCareers.org in search of the information, advice, and opportunities they need to take the next step in their careers.

A complete career resource, free to the public, *Science Careers* offers a suite of tools and services developed specifically for scientists. With hundreds of career development articles, webinars and downloadable booklets filled with practical advice, a community forum providing answers to career questions, and thousands of job listings in academia, government, and industry, *Science Careers* has helped countless individuals prepare themselves for successful careers.

As a AAAS member, your dues help AAAS make this service freely available to the scientific community. If you're not a member, join us. Together we can make a difference.

To learn more, visit aaas.org/plusyou/sciencecareers





The University of Konstanz with its »Institutional Strategy to promote Top-Level Research« has been receiving continuous funding since 2007 within the framework of the Excellence Initiative by the German Federal and State Governments.

The Faculty of Sciences, Department of Chemistry, seeks to fill a

W1-Professorship of Magnetic resonance spectroscopy of complex molecular systems

to be filled as soon as possible.

The research focus of the professorship within nuclear magnetic resonance spectroscopy is in the area of investigations of bio molecules and should complement the research activities of the Department of Chemistry. It should further strengthen and deepen the existing co-operation with the neighbouring disciplines and strengthen the collaborations with the Department of Biology.

Further information (reference number 2014/057) please visit our homepage: <http://www.uni-konstanz.de> or by contacting Mr. Hanns Fahlbusch, e-mail: Hanns.Fahlbusch@uni-konstanz.de or by phone: +49 (0) 7531/88-2413.

Applications comprising a cover letter, curriculum vitae, publication list, a list of grants and awards, details of teaching experience, as well as statements of current research topics, future research directions and interests, copies of academic degrees (see pdf-link below) should be sent electronically **until 30. April 2014** formatted into *one pdf-file* to: Prof-2014-057@uni-konstanz.de.



Young Group Leader Positions In Stem Cell Biology

The Institut Pasteur (Paris, France) announces an international call for candidates wishing to establish independent research groups. As part of the **Revive Laboratory of Excellence** programme on "Stem Cells and Regenerative Biology and Medicine" (www.pasteur.fr/revive), candidates will be integrated into the cutting edge interdisciplinary environment provided by the **Department of Developmental & Stem Cell Biology**. Candidates specializing in the field of stem cells in the context of developmental and cell biology, genetics, epigenetics, regeneration, translational research and ageing are encouraged to apply.

To be eligible, candidates must have defended their PhD on or after May 31, 2006 (women with children are eligible up to 11 yrs after their PhD). Successful candidates will be appointed as head of a group of up to 6 people for a period of 5 years. The budget (up to €1,500,000 over 5 years) includes the salary for the group leader, a three-year postdoctoral position, a technician's position, part-time secretarial assistance, a substantial contribution to running costs and equipment, and access to on-campus facilities including state-of-the-art technology core facilities. Candidates should send their formal applications by E-mail to the Director of Scientific Evaluation, Prof. Alain Israël, at the Institut Pasteur (g5revive@pasteur.fr): **the application deadline is May 31, 2014.**

Short-listed candidates will be contacted for interview in early September 2014 and recruitment decisions announced by October 2014.

Applicants should provide the following (in order) in a single pdf file:

1. A brief introductory letter of motivation, including the name of the proposed group. Candidates are encouraged to contact the coordinator of the Revive programme Shahragim Tajbakhsh (shaht@pasteur.fr).
2. A Curriculum Vitae and a full publication list.
3. A description of past and present research activities (up to 5 pages with 1.5 spacing; Times 11 or Arial 10 font size).
4. The proposed research project (up 10 pages with 1.5 spacing; Times 11 or Arial 10 font size).
5. The names of 3 scientists from whom letters of recommendation can be sought, together with the names of scientists with a potential conflict of interest from whom evaluations should not be requested.

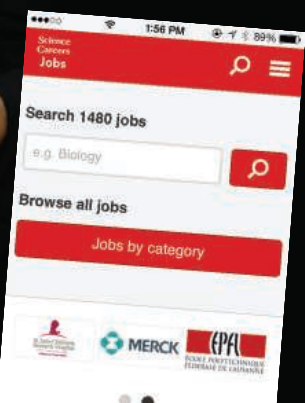
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Science Careers

From the journal Science AAAS

ScienceCareers.org



Prince Philip Professorship of Ecology and Evolutionary Biology

Department of Zoology

The Board of Electors to the Prince Philip Professorship of Ecology and Evolutionary Biology invite applications for this Professorship from persons whose work falls within the general field of the Professorship to take up appointment on 1 October 2014 or as soon as possible thereafter.

Candidates will have an outstanding research record of international stature in the broad field of animal ecology and evolution, and the vision, leadership, experience and enthusiasm to build on current strengths in maintaining and developing a leading research presence. They will hold a PhD or equivalent postgraduate qualification.

Standard professorial duties include teaching and research, examining, supervision and administration. The Professor will be based in Cambridge. A competitive salary will be offered.

Further information is available at:

www.admin.cam.ac.uk/offices/academic/secretary/professorships/ or contact the Academic Secretary, University Offices, The Old Schools, Cambridge, CB2 1TT, (email: ibise@admin.cam.ac.uk).

Applications, consisting of a letter of application, a statement of current and future research plans, a curriculum vitae and a publications list, along with details of three referees should be made online no later than Wednesday 30 April 2014. The Department would particularly welcome applications from women as it has an historic imbalance in the number of women holding academic staff positions.

Informal enquiries may be made to Professor Michael Akam, Head of the Department of Zoology, Cambridge, telephone +44 (0)1223) 336601 or email HOD@zoo.cam.ac.uk.

Please quote reference PF03005 on your application and in any correspondence about this vacancy.

Closing date: 30 April 2014

The University values diversity and is committed to equality of opportunity.

The University has a responsibility to ensure that all employees are eligible to live and work in the UK.

POSITIONS OPEN

ASSISTANT PROFESSOR Immunology and Microbiology

The Department of Microbiology/Immunology at A.T. Still University invites applications for a 12-month, tenure-track position available after July 1, 2014. The successful candidate must demonstrate strong potential to develop an independent research program in an area of immunology or medical microbiology, and be an innovative educator for medical and dental students at our Kirksville, Missouri campus. Applicants must have a doctorate degree and significant postdoctoral research experience. Position details are described at **website: <http://www.hrjobs.org/kcom-assistant-professor-microbiology-and-immunology/job/4433238>**. Send letter of application, curriculum vitae with research interests, and contact information for three references to: **Neil Sargentini, Ph.D., Department Chair, at e-mail: nsargentini@atsu.edu**. *ATSU is an Equal Opportunity Employer and does not discriminate on the basis of race, color, religion, national origin, sex, gender, sexual orientation, age, or disability.*

FOREST GENETICS RESEARCH LEADER

The Washington State Department of Natural Resources is seeking candidates in the fields of molecular genetics, microbiology, mycology, dendrology, or related field to fill a new research position. This position will serve as an expert in the field of forest genetics, independently performing original scientific research to improve the health and productivity of native conifer trees. The position resides in Olympia, Washington, monthly salary up to \$8,167 (depending on qualifications), benefits package, and potential moving expense allowance. For information call **telephone: 360-902-1228** or go to our jobsite at **website: <http://www.dnr.wa.gov/AboutDNR/Employment/Pages/Home.aspx>** scroll down to Forest Genetics Research Leader announcement.

FACULTY POSITIONS - MEDICAL SCHOOL

The Saint James School of Medicine, an international medical school (**website: <http://www.sjsm.org>**), invites applications from candidates with teaching and/or research experience in any of the basic medical sciences for its Caribbean campuses. Faculty positions are currently available in Pathology, Genetics, Neuroscience, and Biochemistry. Applicants must be M.D., and/or Ph.D.

Teaching experience in the U.S. system is desirable but not required. Retired persons are encouraged to apply. Attractive salary and benefits. Submit curriculum vitae to **e-mail: jobs@mail.sjsm.org** or mail to: **HRDS Inc., 1480 Renaissance Drive, Suite 300, Park Ridge, IL 60068.**

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